Plant dispersal in rivers
A mechanistic and molecular approach

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Chapter 1

General Introduction

Plants in rivers: a challenging habitat

Aquatic plants are plants that grow in or on the water, either completely or partly submerged, and that are dependent on the water for some or all of the stages of their life cycle. Based on the habitat requirements necessary to complete their generative cycle, aquatic plants can be divided into hydrophytes, helophytes and pleustohelophytes (Table 1; Den Hartog & Segal 1964). Of all freshwater ecosystems on this planet, river systems present a number of unique challenges to aquatic plants: Firstly, in rivers plants are subjected to turbulent flow which may relentlessly pull and the batter aboveground plant parts and, in very swift flow, may even break off leaves and wash them away (Haslam 1978). The flow may also cause erosion of the soil around plants exposing and possibly damaging their rooting systems, and if the erosion is particularly severe whole plants may become uprooted and be washed downstream (Haslam 1978; Riis & Biggs 2003). Secondly, rivers may pose special constraints on the mode of reproduction (sexual versus asexual) of aquatic plants. Although adapted to the aquatic environment, most aquatic plants still rely on wind- or insect-mediated pollination (Sculthorpe 1967; Large *et al.* 1996). Thus, in order to reproduce sexually, plants have to produce emerging structures, which allow them to flower on or above the water surface. Sometimes, however, the locally reigning environmental conditions (*e.g.* particularly high water velocity, but also deep water or intense shading) prevent the formation of emergent flowering stems and, hence, preclude sexual reproduction (Haslam 1978; Dawson & Kern-Hansen 1978). Nevertheless, since most (if not all) aquatic plants are particularly apt to clonal reproduction (Grace 1993; Barrat-Segretain 1996; Van Groenendael *et al.* 1997), plant populations may still ensure long term population persistence and possibly even population growth by means of clonal expansion, in locations where sexual reproduction is suppressed by environmental conditions (Sculthorpe 1967; Bartley & Spence 1987; Barrett *et al.* 1993; Grace 1993; Honnay & Bossuyt 2005). Finally, riverine environments may pose special constraints on plant dispersal because of two unique characteristics that all river ecosystems share: (i) the one-dimensional, linear arrangement of populations along the river course and (ii) the unidirectional nature of the water flow. These two characteristics are likely to cause an asymmetry in the dispersal among riverine populations (with dispersal occurring predominantly in a downstream direction) and, consequently, a disparity in the influx of propagules among populations, which may be greater in downstream compared to upstream located populations. This disparity may have important consequences for the genetic diversity within populations and the persistence of plant populations.

The importance of dispersal

Dispersal plays a fundamental role in the life-history of plants, affecting the biology, ecology, and genetics of plant populations (*e.g.* Fenner 2000; Silvertown & Antonovics 2001; Silvertown & Charlesworth 2001; Hanski & Gaggiotti 2004). Several reasons have been put forward to explain why seed dispersal may be advantageous for plants: (i) the avoidance of disproportionate seedling mortality near the parent plant, (ii) the colonisation of empty suitable habitats, which is particularly

Table 1 Classification of plants associated with the aquatic environment (Den Hartog & Segal 1964).

1. Hydrophytes	Plants that are able to achieve their generative cycle when all vegetative parts are submerged or are supported by the water (floating leaves), or which occur normally submerged but are induced to reproduce sexually when their vegetative parts are dying due to emersion.
2. Helophytes	Plants which root in the bottom and of which the basal parts are submerged almost continuously, but whose leaves and inflorescences rise above the water surface (*e.g. Sparganium emersum* and *Sagittaria sagittifolia*).
3. Pleustohelophytes	Plants drifting freely on the surface with submerged root systems, but with all other vegetative parts and inflorescences rising above the water, due to their aerenchymatic structure.

important in habitats characterized by high levels of disturbance, such as in metapopulations which are characterized by a population turnover and therefore critically depend on continuous recolonizations for their persistence, (iii) the range expansion of species, which plays a particularly important role in biological invasions, and (iv) the exchange of genetic information among populations, which plays an important role in the population genetics (*e.g.* in determining the size of the genetic neighbourhood of plants), conservation genetics (*e.g.* in preventing inbreeding) and evolutionary potential of plants (Howe & Smallwood 1982; Silvertown & Antonovics 2001; Hanski & Gaggiotti 2004; Ouborg *et al.* 2006).

Plants stand still but their propagules don't – The propagules, mechanisms and patterns of plant dispersal in rivers

Spatial connectivity along rivers is one of the most important factors determining the distribution of aquatic plants in lowland rivers (Demars & Harper 2005). However, plants face a momentous difficulty in that most of them lead a sessile life style and cannot move from one location to another (the pleustohelophytes posing a notable exception; Table 1). To overcome this problem, plants have principally three different types of propagules at their disposal with which to accomplish their dispersal (Table 2): pollen (which carry the genetic information of the father only), generative propagules (*i.e.* seeds; which carry the genetic information of both the father and mother) and vegetative propagules (*i.e.* tubers, turions, bulbils, stolons, rhizomes and viable plant fragments; which are genetically identical to the parent plant) (Bartley & Spence 1987; Barrat-Segretain 1996).

Most authors discern three principal mechanisms that may play a role in the dispersal of these different types of propagules: wind dispersal (anemochory), water dispersal (hydrochory) and animal dispersal (zoochory) (Table 2; Ridley 1930; Van der Pijl 1972; Cook 1988; Barrat-Segretain 1996). Although wind is an important agent of seed dispersal among terrestrial plants, it hardly seems to play a role in the dispersal of either seeds or vegetative propagules of most aquatic plants (Cook 1988; Barrat-Segretain 1996). This lack of importance of wind dispersal is generally attributed to the higher risk, for both seeds and vegetative propagules, of being blown to terrestrial sites on the

Table 2 Different agents, mechanisms, propagules and directions of plant dispersal in rivers [++ = likely vector of dispersal, + = possible vector of dispersal; - = unlikely vector of dispersal; i = internal animal-mediated dispersal (endozoochory); e = external dispersal (ectozoochory)].

		Propagules			Direction of dispersal in river systems			
Dispersal agents	Dispersal mechanisms	Pollen[a]	Seeds[b]	Vegetative propagules[c]	Up-stream	Trans-versal	Down-stream	Over-land[e]
Wind	Anemochory	++	-	-	yes	yes	yes	yes
Insects	Insectochory	++	-	-	yes	yes	yes	yes
Water	Hydrochory	-	++	++	-	yes[d]	yes	-
Fishes	Ichthyochory	-	++(i)	-	yes	yes[d]	yes	-
Waterfowl	Ornithochory	-	++(i,e)	+(e)	yes	yes	yes	yes
Mammals	Mammaliochory	-	++(i,e)	+(e)	yes	yes	yes	yes

[a] Dispersal of male genes only (note that pollen dispersal alone, cannot lead to the colonization of empty habitats).
[b] Dispersal of generative propagules.
[c] Dispersal of vegetative propagules (genetically identical to the mother plant).
[d] Transversal dispersal (*i.e.* to oxbow and floodplain lakes) only possible during large flood events.
[e] Overland dispersal (*i.e.* to nearly lakes or river catchments).

surrounding land rather than to aquatic sites in the river (Cook 1988; Barrat-Segretain 1996). On the other hand, wind does play a role in the pollen dispersal of aquatic plant species (anemophily; Cook 1988). In contrast, water is a very important dispersal agent for seeds and vegetative plant fragments (hydrochory) of many aquatic plants, while it hardly plays a role in the dispersal of the pollen (hydrophily) of freshwater plants (although it does play a role in the pollen dispersal of marine algae and seagrasses) (Sculthorpe 1967; Cook 1988; Barrat-Segretain 1996; Boedeltje 2005; Riis & Sand-Jensen 2006). Seeds often fall directly into the water (or sometimes onto the river bank close to the water, where they may later be transported to the main channel during flood events) and, both seeds and vegetative propagules, are often positively buoyant facilitating their long-distance dispersal. Finally, the importance of zoochory in the dispersal of (predominantly seeds of) aquatic plants was already recognised a long time ago (Darwin 1859; Ridley 1930; Sculthrophe 1967). The diet of many water associated animals (*e.g.* fish, water birds and mammals) consists (partly) of seeds of aquatic plants, which may be internally transported to, and subsequently defaecated in, new locations (Ridley 1930; Van der Pijl 1972). Alternatively, seeds may adhere to the fur, feathers or feet of animals and thus be transported (Sorensen 1986; Cook 1990; Smith & Stiles 1994; Figuerola & Green 2002). In addition, insects may play a role in the dispersal of pollen of aquatic plants (entomophily). Notably, propagules may potentially be dispersed by multiple dispersal mechanisms, with each type of propagule and each dispersal mechanism leading to different rates, distances and directions of dispersal (see Table 2; Portnoy & Willson 1993; Willson 1993; Eriksson & Jakobsson 1999).

The probability of seed deposition typically follows a leptokurtic curve with increasing distance away from the parent plant, regardless of the propagule type or mechanism of dispersal (Strykstra *et al.* 1998; Nathan & Muller-Landau 2000; Ouborg & Eriksson 2004); *i.e.* no or very little seed deposition in close proximity to the parent, then rapidly increasing to maximum seed

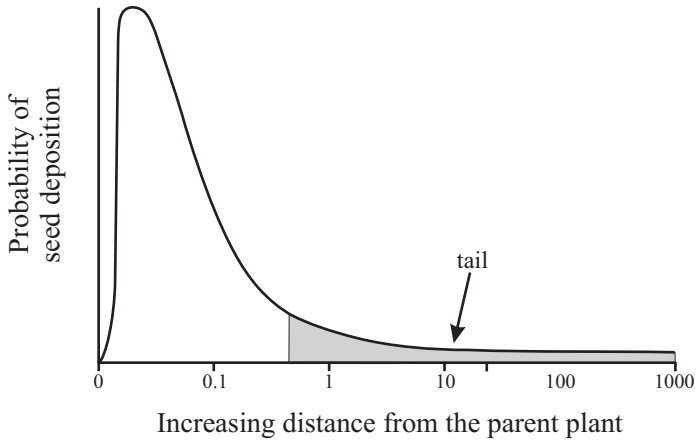

Fig. 1 Leptokurtic probability curve of seed deposition away from a single seed source (*i.e.* parent plant). The tail of the distribution may be particularly hard to model, because it often depends on stochastic, unpredictable and non-standard dispersal events (Portnoy & Willson 1993; Strykstra *et al.* 1998; Higgins *et al.* 2003).

deposition followed by a gradual decrease in seed deposition with increasing distance, the manner in which the probability of seed deposition decreases often approximated by a negative exponential function (Fig. 1; Portnoy & Willson 1993; Willson 1993; Strykstra *et al.* 1998). Although long-distance dispersal (LDD) will be relatively rare (as modelled by the tail of the dispersal curve) it may be particularly important in biological processes that take place on larger spatial scales, such as the evolution of populations, metapopulation dynamics, biological invasions and the diversity of ecological communities (Cain *et al.* 2000; Nathan *et al.* 2003; Higgins *et al.* 2003; Muller-Landau *et al.* 2003; Nathan 2006). Note that, although seed deposition follows a leptokurtic curve regardless of the type or mechanism of dispersal, the *shape* of the dispersal kernel may differ greatly among different types of propagules or mechanisms of dispersal.

Plant dispersal and the regional dynamics of plant populations

Spatial structure and regional dynamics of plant populations

Dispersal has a large bearing on the spatial structure and regional dynamics of plant populations (Eriksson 1996; Husband & Barrett 1996; Harrison & Taylor 1997; Freckleton & Watkinson 2002; Ouborg & Eriksson 2004). According to Freckleton & Watkinson (2002), a set of local plant populations at regional scales can be classified into either of three groups: *spatially extended populations*, *metapopulations* or *regional ensembles*.

Spatially extended populations exist as plant clumps or plant patches in a single population that is dominated by local processes (births and deaths) and in which patchiness arises as a consequence of local disturbances. Three different types of spatially extended populations are distinguished: (i) an extended population, *i.e.* a population that is distributed almost continuously across a large area of suitable habitat (Fig. 2a); (ii) a patchy population, *i.e.* a population that exists as a series of distinct plant clumps or patches in a large area of suitable habitat (Fig. 2b); (iii) a spatially structured

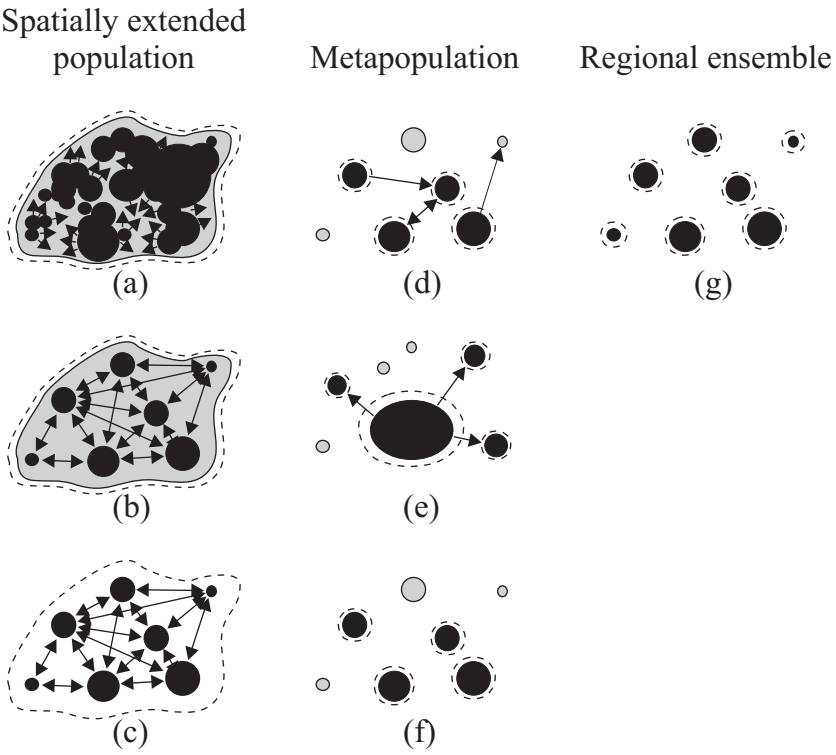

Fig. 2 Classification of the spatial structure and regional dynamics of plant populations (Harrison & Taylor 1997; Freckleton & Watkinson 2002). *Spatially extended populations*: **(a)** extended population, **(b)** patchy population and **(c)** spatially structured local population. *Metapopulations*: **(d)** classic metapopulation, **(e)** source-sink or mainland-island metapopulation and **(f)** non-equilibrium metapopulation. *Regional ensembles*: **(g)** remnant, shifting cloud or island populations (black circles, occupied habitat patches; grey circles, vacant habitat patches; dotted lines, boundaries of local populations; solid lines, boundaries of suitable habitat; arrows, dispersal).

local population, *i.e.* a populations that exists as a series of distinct plant patches in an area where suitable habitats is distributed in discrete patches (Fig. 2c). *Metapopulations* exist as a series of local populations dominated by regional processes (population extinction, interpopulation dispersal and recolonization). In metapopulations, suitable habitat typically occurs as discrete patches within a larger matrix of unsuitable habitat, with limited migration among patches. Also here three different types of metapopulations are distinguished: (i) a classic metapopulation (levins 1969), *i.e.* in which all habitat patches have an equal probability of being colonized and becoming extinct (Fig. 2d); (ii) a source-sink or mainland-island metapopulation, *i.e.* in which most habitat patches are not capable of maintaining persistent populations (sinks), but instead rely on a continuous immigration from larger, more persistent, source populations (Fig. 2e); (iii) a non-equilibrium metapopulation (Harrison & Taylor 1997), *i.e.* in which the balance between local extinctions and recolonizations is disrupted (typically as the species' habitat is undergoing fragmentation). In non-equilibrium metapopulations the rate of local extinction often increases while the rate of recolonization is declining, ultimately leading to the extinction of the entire metapopulation (Fig. 2f). *Regional ensembles* consist of a regional set of highly persistent (*i.e.* little or no local extinction) and basically unconnected (*i.e.* no migration) populations, in which the size and persistence of populations are entirely determined by local processes (Fig. 2g; based on the presence of seed banks and/or the difficulty of identifying

suitable habitat in the field regional ensembles may further be divided into remnant populations, shifting cloud populations or island populations; see Freckleton & Watkinson 2002).

Plant-specific problems with the metapopulation concept

Although the metapopulation concept has proven to be a very useful concept in animal studies (*e.g.* Gilpin & Hanski 1991; Hanski & Gilpin 1997; Hanski 1999; Hanski & Gaggiotti 2004), its application to questions concerning the spatial structure of plant populations has recently been under debate (Eriksson 1996; Husband & Barrett 1996; Bullock *et al.* 2002; Freckleton & Watkinson 2002, 2003; Ehrlén & Eriksson 2003; Ouborg & Eriksson 2004; Murphy & Lovett-Doust 2004). Several of these authors have listed a number of practical problems that make a distinction between spatially extended populations, metapopulations and regional ensembles, based on field observations only, very difficult.

Metapopulations are often modelled as suitable habitat patches in a matrix of unsuitable habitat, however, in the field the distinction between suitable habitat (the patches) and unsuitable habitat (the matrix) may be difficult to make (Freckleton & Watkinson 2002). For most plants, suitable habitat is defined by resource quality and environmental conditions, both of which typically occur as gradients in nature. As a result suitable habitat is more likely to exist as a continuum for most plants, from optimal habitat to suitable habitat to sub-optimal habitat, rather than discrete suitable patches in a hostile matrix (Cox & Moore 1980; Murphy & Lovett-Doust 2004). Moreover, the suitability of habitat patches may change over time, due to changes in the local environmental conditions. The existence of long-lived life stages (particularly in clonal plants) make it difficult to determine the suitability of habitat patches, because here the presence of plants does not necessarily mean that habitat patches are (still) suitable. For example, clonal reproduction in a habitat patch may allow local populations to exist for a long time, even though that habitat patch has become unsuitable and does not allow sexual reproduction and/or seedling recruitment anymore (Murphy & Lovett-Doust 2004; Ouborg & Eriksson 2004). However, Pannell & Obbard (2003) argued that, when applying the metapopulation concept to population genetic studies, it is not the spatial distribution of suitable habitat that counts but rather the discrete nature of the groups (populations) of the organisms that are being studied.

The long life spans of plants may make the identification of metapopulation structure difficult for another reason. In habitats that are undergoing severe reduction in patch densities and, as a result, an increase in mean interpatch distance (due to habitat fragmentation), the (re)colonization rate will rapidly decrease potentially leading to non-equilibrium conditions (Hanski 1999; Ouborg & Eriksson 2004). Here, local populations may continue to exist for a long time, displaying sexual reproduction and local recruitment (but little or no interpopulation dispersal anymore), giving the appearance of a metapopulation structure (Fig. 2d) while in fact the populations exist as a non-equilibrium metapopulation deemed for extinction (Fig. 2f).

Another problem specific to plant populations is the potential presence of long-lived seed banks. As a result, some patches may appear to be empty, while the plant species may nevertheless

be present as a persistent seed bank waiting for favourable conditions to restart population growth (Eriksson 1996). The presence of a viable, yet not obviously visible, seed bank may therefore hamper the assessment of patch occupancy in the field (Ouborg & Eriksson 2004).

Finally, although dispersal and subsequent establishment are essential processes in metapopulations, it is difficult to obtain direct estimates of plant migration rates and distances in the field (see also below), and very difficult to distinguish between seedling recruitment due to emergence from the local seed bank (*i.e.* resulting from local sexual reproduction) or due to immigration of seeds from other populations.

Different approaches for studying plant dispersal in rivers

While seed dispersal is a very important biological process, it is also very difficult to quantify. Several different approaches have been used to quantify plant dispersal in river systems, which can be roughly divided into empirical, mechanistic and molecular approaches (Ouborg *et al.* 1999; Ouborg & Eriksson 2004). Firstly, empirical approaches assess the amount and distance of seed dispersal directly in the field by means of trapping seeds (Skoglund 1990; Middelton 1995; Goodson *et al.* 2003; Wolters *et al.* 2004), seed mimics (Nilsson *et al.* 1991; Andersson *et al.* 2000; Levine 2001) or vegetative propagules (Johansson & Nilsson 1993; Boedeltje *et al.* 2004) at various distances from source plants and subsequently constructing frequency-distance distributions (Craddock & Huenneke 1997; Strykstra *et al.* 1998). Secondly, mechanistic approaches assess the dispersal characteristics of seeds under controlled (experimental) conditions and relate this information to the putative dispersal agents in order to construct predictive models of seed dispersal (Nathan & Muller-Landau 2000). The best known examples stem studies on terrestrial plants, where wind-tunnel experiments are used to assess the terminal velocity of seeds in order to model anemochoric seed dispersal patterns under given model parameters (*e.g.* release heights, wind direction and velocity; Van Dorp *et al.* 1996; Nathan *et al.* 2002; Soons *et al.* 2004). Likewise, in aquatic systems, buoyancy experiments may be used to assess the floating duration of seeds (Staniforth & Cavers 1976; Smits *et al.* 1989; Hroudova *et al.* 1997; Van den Broek *et al.* 2005) in order to model hydrochoric seed dispersal patterns under given current velocities (Merritt & Wohl 2002) or to explain observed vegetation patterns in the field (Hart & Cox 1995; Danvind & Nilsson 1997), while feeding experiments may be used to model animal-mediated seed dispersal (*i.e.* endozoochory) (Pakeman *et al.* 2001; Charalambidou & Santamaría 2002). Finally, molecular approaches assess the distribution of genetic variation within and among populations, in order to make inferences about the rate of gene flow that has occurred between them (Ouborg *et al.* 1999; Cain *et al.* 2000). These approaches rely on the use of highly variable, neutral, genetic markers (*e.g.* allozymes, RFLPs, RAPDs, AFLPs, microsatellites, and DNA sequences of non-coding regions) in combination with sophisticated software for the analyses of population genetic data, and may yield estimates of the rates, distances and directions of dispersal. Since the three approaches described above will each provide different information about the dispersal process of plants, it has been advised to use more than one of these approaches when studying plant dispersal (Ouborg & Eriksson 2004).

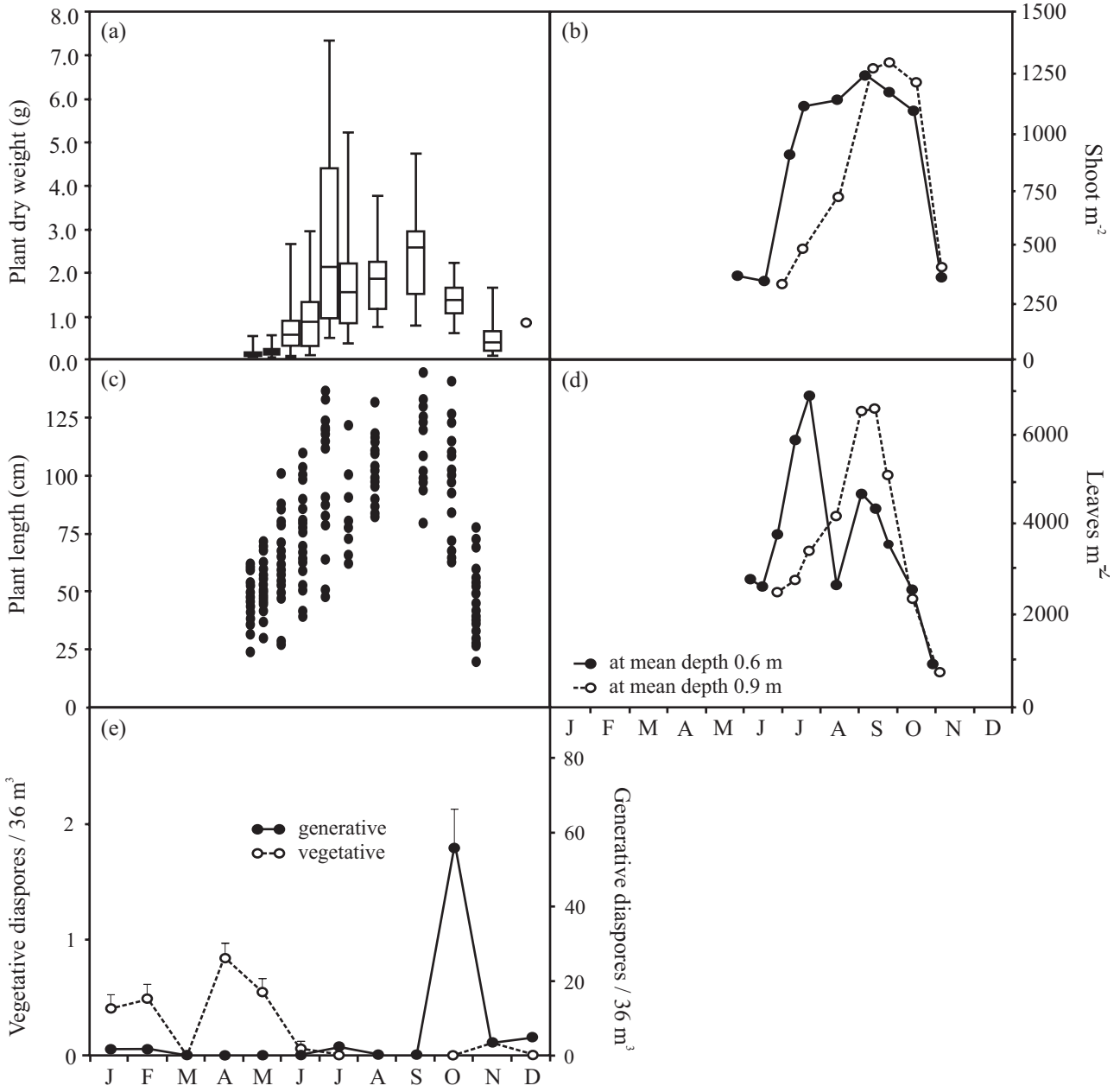

Fig. 3 Seasonal variation in **(a)** plant dry weight, **(b)** shoot density, **(c)** plant length, **(d)** leaf density, and **(e)** dispersal phenology of *Sparganium emersum*. (a-c: river Eider, Northwest Germany, redrawn after Trepel *et al.* 2003; b-d: river Suså, Denmark, redrawn after Wiggers-Nielsen *et al.* 1985; e: Twentekanaal, the Netherlands, redrawn after Boedeltje *et al.* 2004).

Study species

The general aim of this thesis was to investigate plant dispersal in river systems. We selected the unbranched burreed (*Sparganium emersum* Rehmann 1871 = *S. simplex* Hudson 1778; Sparganiaceae) as our study organism, because this species has a number of interesting characteristics that make it a very suitable species for studying several aspects of plant dispersal in rivers: it is a facultatively

clonal species (capable of both sexual and asexual reproduction), it has different propagules of dispersal (pollen, seeds and vegetative plant fragments) which may potentially be dispersed by different vectors (wind, water, animals), it is a key species of one of the most important plant associations found in north-west European lowland rivers, the *Sparganieto-Sagittarietum* association (*Sagittaria sagittifolia* being the other key species of this association; Cook & Nicholls 1986; Weeda *et al.* 2001), and finally, it is one of the most common species found in our lowland rivers which increases the likelihood of finding enough populations, with sufficient individuals, for a sound design of our mechanistic and genetic studies.

S. emersum is an aquatic vascular macrophyte (helophyte, Table 1) that is widely distributed throughout Eurasia and North America (Cook & Nicholls 1986). It typically grows in a narrow band at the margins of rivers, streams and canals that are characterized by shallow, slow flowing, nutrient-rich waters (Haslam 1978; Van der Meijden 1990) with soft, clay and sandy bottoms which provide optimal conditions for the deep-rooting rhizomes of *S. emersum* (Haslam 1978; De Lyon & Roelofs 1986; Baattrup-Pedersen & Riis 1999; Riiss *et al.* 2000). Like many other temperate aquatic and riparian plant species, *S. emersum* survives the winter period as rhizomes (*i.e.* root structures stored with starch) buried underground (Kausch *et al.* 1981; Wiggers-Nielsen *et al.* 1985; Trepel *et al.* 2003). In spring, when the water temperatures in the river rise above 10ºC, plants will resprout from the buried rhizomes and display a rapid growth (showing a rapid increase in biomass, plant length and shoot and leaf densities; Fig. 3a-d). During late summer *S. emersum* will re-allocate its energy to the production of flowers. *S. emersum* is a monoecious species, but with temporally separated male and female flowers (pollen dispersal preceding sigma receptivity). In autumn, the seeds will be released (falling into the water) and be dispersed by water currents (Boedeltje *et al.* 2004). Literature shows that their seeds are ingested by a number of fish and waterfowl species, offering the possibility of internal animal-mediated seed dispersal (McAtee 1918; Ridley 1930; Anderson 1959). Moreover, experimental studies have shown that vegetative plant fragments of *S. emersum* remain viable and capable of establishment even after floating for up to 10 weeks (Barrat-Segretain & Amoros, 1996; Barrat-Segretain *et al.* 1998, 1999; Barrat-Segretain & Bornette 2000), potentially offering an important mechanism of dispersal. However, empirical studies suggest that, for *S. emersum*, the dispersal of vegetative plant fragments may be less important compared to the dispersal of seeds (Boedeltje *et al.* 2004; Fig. 3e). Finally, in late autumn, when water temperatures fall below 10ºC, the aboveground plant parts will rapidly die off and the energy will, once again, be stored in the belowground rhizomes.

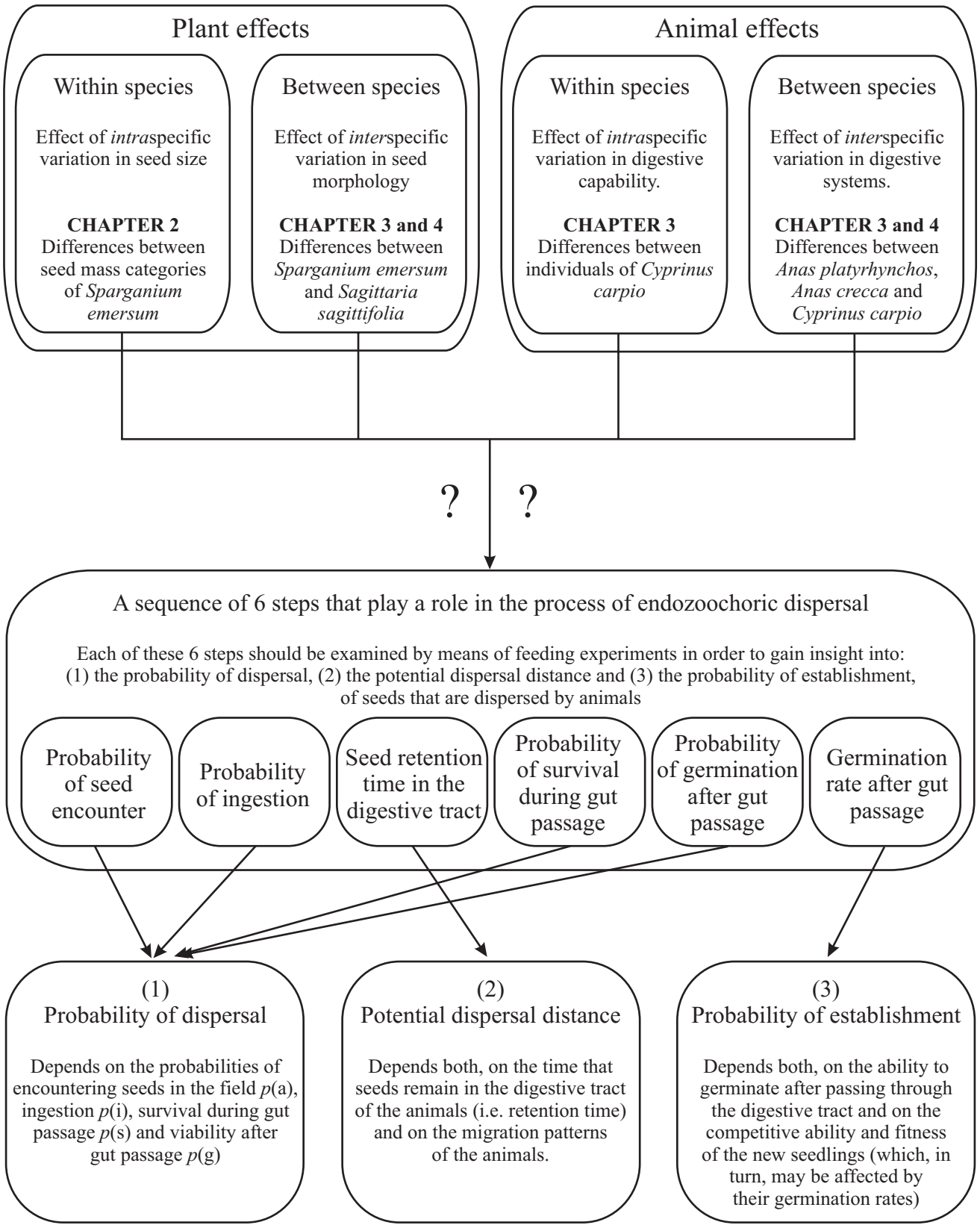

Fig. 4 Schematic representation of the various aspects of the endozoochoric dispersal process that have been studied in this thesis. Intra- and interspecific variation in seed characteristics (plant effects) and digestive capabilities (animal effects) will affect the sequential steps that play a role in the endozoochoric dispersal process. This, in turn, will affect the probability of seed dispersal, the potential dispersal distance and the probability of establishment.

Research questions and outline of this thesis

The aim of this thesis is to investigate the dispersal of *S. emersum* in river systems, using both a mechanistic (experimental) and a molecular (population genetic) approach. The main questions that are addressed in the present thesis have been listed below.

1. Variability in environmental conditions may lead to phenotypic variation among plants, potentially affecting their mode of reproduction (sexual versus clonal). In this thesis we use field observations as well as a molecular approach to assess how spatial variation in local environmental conditions (such as water depth, current velocity and shading) affect the phenotype of *S. emersum*, how this in turn affects its mode of reproduction within populations, and ultimately how this translates into genotypic diversity within populations (**Chapters 5-6**).

2. The importance of hydrochory for plant dispersal has received considerable attention, having been studied mainly by using an empirical approach. In this thesis we apply a mechanistic approach to study the potential for long-distance hydrochorous dispersal in *S. emersum*, and a molecular approach to assess the relative contribution of seed versus vegetative leaf fragments in the effective dispersal (dispersal and successful establishment) of *S. emersum* in three different river systems (**Chapters 5-8**).

3. Although zoochory may play an important role in the dispersal of plants, its importance in aquatic ecosystems has received relatively little scientific attention (especially when compared to terrestrial systems). In this thesis we apply a mechanistic approach to study the potential for internal seed dispersal of *S. emersum* and *Sagittaria sagittifolia* by fish and waterfowl. More specifically, we examined how variation within plant species (effect of seed size), among plant species (effect of seed structure or morphology), within animal species (effect of 'genotypic' variation in digestive capabilities among individuals) and among animal species (effect of interspecific differences in the structure of the digestive system) affect seed dispersal (summarized in Fig. 4) (**Chapters 2-4**).

4. The nature of a dispersal vector may have a large bearing on the dispersal of seeds. In this thesis we use a mechanistic approach to compare the direction and potential distance of dispersal, as well as the shape of the dispersal curve (Fig. 1), of *S. emersum* seeds that are dispersed by three different dispersal agents (water, fish and waterfowl) (**Chapters 2-4 and 8**).

5. The unidirectional nature of flow in rivers may lead to an asymmetry in the migration rates between populations (with respect to upstream versus downstream directions), which in turn may affect the pattern of genetic diversity within populations along the course of a river (potentially leading to lower genetic diversities in upstream located populations and higher genetic diversities in downstream populations). In this thesis we use a molecular approach to assess whether there is an asymmetry in the dispersal (gene flow) among populations and whether this affects the pattern of genetic diversity within populations along three different river systems (**Chapters 6-7**).

6. As mentioned above, the ascription of natural plant populations to Freckleton & Watkinson's (2002) classification of three main types of spatial structure and regional dynamics (*i.e.* spatially extended population, metapopulation, regional ensemble), based only on field observations, may be very difficult. A molecular approach may be helpful in distinguishing between these three models of regional population structure. To this end we propose a number of testable hypotheses about the genetic structure and the rate of gene flow among populations that may be used to assess the regional structure of plants populations (**Chapter 7**). We then used these testable hypotheses to assess the regional structure of *S. emersum* populations in three different river systems (**Chapters 6-8**).

7. Aquatic organisms that inhabit river systems are continuously facing the danger of being swept away to downstream areas. A long-standing theory, dubbed 'the drift paradox', states that aquatic organisms will not be able to persist in the upper reaches of one-dimensional linear ecosystems, characterized by unidirectional flow, if the organisms cannot advance upstream against the flow in order to recolonized depopulated areas (Speirs & Gurney 2001; Humphries & Ruxton 2002; Pachepsky *et al.* 2005). Surprisingly, this theory has only been addressed for aquatic invertebrates that are able to actively move upstream (crawling, swimming, flying), while the drift paradox has never been studied for sessile organisms (*e.g.* most aquatic plants and a number of bivalve species) which lack any means of active upstream migration. In this thesis, we use a molecular approach to examine whether plants 'are able to move upstream', as well as to assess the rate of upstream migration (**Chapter 7**). Furthermore, we use a mechanistic approach to examine the potential mechanisms of upstream dispersal (**Chapters 2-4**). Finally, we discuss the persistence of plant populations in river ecosystems in the light of the drift paradox (**Chapter 4 and 8**).

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Chapter 2

Intraspecific variation in seed size does not affect the dispersal of *Sparganium emersum* by fish

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(submitted)

Summary

Variation in phenotypic seed traits will affect the probability of animal-assisted seed dispersal, both within and between plant species. In this study, we examine how intraspecific variation in seed size in the unbranched burreed (Sparganium emersum) affects the probability of ingestion, retention time, survival rate during gut passage, and viability and germination rate after gut passage, when fed to common carp (Cyprinus carpio). Our results, firstly, revealed a significant negative relationship between seed size and seed ingestion, which was counterbalanced by an equally strong but positive relationship between seed size and seed survival during gut passage. A relationship between seed size and seed viability after gut passage was not found. Consequently, overall, the probability of dispersal did not differ between seed sizes. Secondly, seed size did not affect the time that seeds remained in the digestive tract of carp, suggesting that the potential dispersal distance will also not differ between seed sizes. Finally, under controlled conditions we found a small, though significant, negative effect of seed size on germination rate after gut passage. However, arguably, this effect might be too small to be translated into competitive (dis)advantages among conspecific seedlings under natural conditions in the field. Together, these data suggest that there are little or no differences in the probability of dispersal, the potential dispersal distance and competitive abilities after establishment of differently sized S. emersum seeds that are dispersed by fish. Moreover, this research highlights the importance of studying all stages of the endozoochorous dispersal process in order to estimate the effect of a phenotypic seed trait on plant dispersal.

Introduction

Seed dispersal is assumed to have important fitness advantages for plants, by reducing density-dependent mortality (Escape hypothesis) and increasing the chances of founding a lineage in a new locality (Colonization hypothesis) (Howe and Smallwood 1982), and plays a fundamental role in (meta)population ecology, population genetics and evolutionary biology of plants (Ouborg *et al.* 1999; Ouborg and Eriksson 2004; Pollux *et al.* in press). Most plant species have a sessile life-style and rely on a variety of vectors to disperse their seeds: *e.g.* wind (anemochory), water (hydrochory) and animals (zoochory) (Ridly 1930; Van der Pijl 1982; Higgins *et al.* 2003).

In aquatic environments internal seed dispersal is considered to be an important mode of plant dispersal (Barrat-Segretain 1996; Figuerola and Green 2002). The dispersal probability of seeds that are internally dispersed by animals (endozoochory) depends on a number of processes that can be separated into a sequence of steps (Charalambidou & Santamaría 2002): (i) the probability that seeds are ingested (Alcántara and Rey 2003; Gómez 2004), which, in turn, is related to both the relative availability of seeds in the field and the feeding preferences of the animals (Jordano 1995 2000; Celis-Diez *et al.* 2004; Bruun and Poschlod 2006); (ii) the time seeds are retained in the digestive system (*i.e.* retention time), which together with disperser movements affects the potential distance and direction of dispersal (Jordano 2000; Stiles 2000; Higgins *et al.* 2003; Westcott *et al.* 2005); (iii) the resistance of seeds against digestion in the intestinal tract, which determines the probability that seeds survive a passage through the intestinal tract of animal dispersers (Gardener *et al.* 1993a,b; Charalambidou and Santamaría 2002; Pollux *et al.* 2005); and (iv) the viability and germination rate of seeds after gut passage, which may be decreased, enhanced or unaffected (Traveset 1998), and determines the probability of germination and successful establishment of the defecated seeds (Traveset 1998; Traveset and Verdú 2002; Charalambidou and Santamaría 2002).

Phenotypic seed traits (*e.g.* seed size, shape, seed coat hardness and the presence of external structure; Harper *et al.* 1970) will have different impacts on each of these sequential steps in the dispersal process: for example, a positive effect of a phenotypic trait on seed ingestion may be nullified by negative effects on seed survival and seed viability, resulting in an unexpected relationship between phenotypic seed traits and the overall dispersal success of seeds (Gómez 2004; Pollux *et al.* 2006). Thus, when studying the effect of a phenotypic seed trait on the dispersal success of animal dispersed seeds, it is essential to study the complete sequence of steps that affect the probability of seed dispersal (Charalambidou & Santamaría 2002).

Seed size is a phenotypic seed trait that varies widely within and between plant species (*e.g.* Harper *et al.* 1970; Michaels *et al.* 1988). Comparative studies have shown that variation in seed size, both within and between species, affects the ingestion, retention time, survival rate and viability of seeds passing through the digestive tracts of vertebrates, and hence affects the dispersal probability of seeds. For instance, larger seeds are generally preferred by animals because of their higher profitability in energetic and/or nutrient content (Brewer 2001; Celis-Diez *et al.* 2004), but increased handling costs and anatomical constraints in gape-limited animals may limit the upper range of seed sizes that are ingested (Wheelwright 1985; Levey 1987; Herrera 1995; Jordano 1995 2000; Stevenson *et al.* 2005). Furthermore, small-seeded plant species may have a higher survival

rate than large-seeded species when ingested by large herbivores, because the latter have a higher probability of sustaining mechanical damage during chewing (*i.e.* grinding action by the molar teeth; Pakeman *et al.* 2002; Mouissie *et al.* 2005); while large and heavy seeds may have shorter retention times in the digestive tract of vertebrates, being defaecated more quickly than small and light seeds, potentially leading to higher survival rates yet shorter dispersal distances (Traveset 1998, and references therein). Finally, seed size has also been shown to affect (either positively or negatively) the viability of seeds after passing through the intestinal tracts of vertebrates, although this effect may vary widely between species (Traveset 1998; Traveset and Verdú 2002).

In this study we performed a series of controlled feeding experiments to evaluate the effect of seed size variation within the aquatic macrophyte *Sparganium emersum* (Rehmann 1872, Sparganiaceae) on the probability of dispersal by the common carp (*Cyprinus carpio*). We compared the dispersal probabilities of differently sized seeds by assessing the effect of seed size on each of the different steps in the process of endozoochorous dispersal: (i) the probability of seed ingestion, (ii) the seed retention time, (iii) the probability of seeds survival during gut passage, (iv) the probability of seed germination after gut passage, and (v) the germination rate after gut passage. We hypothesized that seed size would have different (potentially conflicting) effects on each of these dispersal components, and we were particularly interested how this would translate into differences in the dispersal probability between differently sized seeds.

Material & Methods

Study species

The unbranched burreed (*Sparganium emersum*) is an aquatic macrophyte that is widely distributed along canals and lowland streams throughout Eurasia and North America (Cook and Nicholls 1986). The drupe-like fruit of *S. emersum* consists of a seed enclosed in a hard scleridial endocarp and a tough spongy mesocarp, with a plugged pointy micropyle (Cook 1962; Cook and Nicholls 1986). The common carp (*Cyprinus carpio*) is one of the most widely spread freshwater fish species in the world, commonly found in lakes, canals and lowland rivers in temperate and tropical regions of Eurasia, North America, Africa and Australia. Dietary studies on field-collected individuals have shown that *C. carpio* is an opportunistic omnivorous forager that includes macrophyte seeds in its diet (Ridley 1930; Crivelli 1981; Bergers 1991; García-Berthou 2001). *C. carpio* and *S. emersum* overlap in their distribution, and it has been shown that seeds of *S. emersum* are dispersed by *C. carpio* (Hochreutiner 1899; reference taken from Ridley 1930).

Experimental design

Ripe seeds of *S. emersum* were collected during October 2003 from 75 plants in 3 natural populations along the River Rur (Germany - the Netherlands; 50°57'30"N-6°17'34"E, 50°02'02"N-6°13'55"E and 51°10'53"N-5°59'32"E). To determine the intra-specific variation in seed mass, the fresh

weights of a total of 6463 *S. emersum* seeds were individually measured on a microbalance. Potential relationships between seed mass and seed size related traits were determined by measuring the length and width of 693 randomly selected *S. emersum* seeds and relating this information to their corresponding seed mass. Prior to the feeding experiments, the seeds were stored in glass jars filled with tap water, in a dark cold room at 5 ± 1 ºC, to mimic natural stratifications of Central-North European winters.

Twelve common carp with a mean mass of 0.307 ± 0.045 (SD) kg were obtained from Ruud Vonk Fish Hatchery (Maurik, the Netherlands) in October 2003. The fish were individually kept in 100-L tanks in the fish facilities of Radboud University Nijmegen (the Netherlands), and daily fed on a stable diet of commercial pellets (Trouvit, Trouw & Co, Putten, the Netherlands) amounting to 1 % of their body mass. The water in the tanks was maintained at 24ºC and was continuously aerated and refreshed (50 l h^{-1}). To ensure homogenisation of water quality among the twelve tanks, all tanks were supplied with fresh water coming from the same filtering system.

To test the effect of seed mass of *S. emersum* seeds on the probability of their dispersal, three (repeated) feeding trials were performed. In each feeding trial, the 12 carp were fed 50 *S. emersum* seeds, though each time seeds with a different seed mass: either (L) 'light' seeds < 10 mg, (M) 'medium' seeds 10-20 mg, or (H) 'heavy' seeds > 20 mg (see Fig. 1 for distribution of seed weights in natural populations). To exclude possible effects of the order of seed ingestion, the order in which the L, M and H seeds were fed to the carp was partitioned in a randomized complete block design (RCB; with three blocks).

The three feeding trials were performed in April and May 2004, at weekly intervals. At the beginning of a feeding trial, each of the 12 carp was fed a total of 10 Trouvit food pellets (containing a total of 50 randomly selected seeds of a particular seed mass, *i.e.* L, M or H). Five to ten minutes after feeding, non-ingested seeds (*i.e.* seeds that were expelled by 'spitting'; Sibbing, Osse & Terlouw 1986) were removed from the tanks with aquarium nets (gape size 10x15 cm; square mesh size 1 mm) and counted. Next, for a period of 24 hours faeces were collected every 2 hours from the bottom of the tanks by means of aquarium nets (preliminary tests, lasting 48 hours, showed that the fish always excreted all non-digested seeds well within 24 hours). Collected faeces were immediately rinsed with tap water and sieved using a 500 µm square mesh size sieve (diameter 19 cm). Retrieved seeds were transferred to plastic containers (100 ml) filled with tap water and returned to the dark cold room (5 ± 1 ºC) for the remainder of the experiments to ensure an equal stratification period for all seeds obtained from the three feeding experiments (from seed collection in the field in October 2003 to the germination test in may 2004). For each seed mass, three batches of 50 randomly selected non-ingested seeds were used as controls in the germination experiment. These control seeds received a similar pre- and post-experimental treatment as the seeds used in the feeding experiments (*i.e.* placed in soft pellets soaked in water, sieved with tap water and stored at 5 ± 1 ºC for the remainder of the feeding experiments) to exclude possible effects of pre- or post-feeding treatment of the seeds.

In May 2004, all retrieved and (non-ingested) control seeds were simultaneously set to germinate in a climate chamber with a photoperiod of 16L/8D, a daytime irradiance of 200 µmol photons s^{-1} m^{-2} and a day/night temperature cycle of 25/18 ºC. Seeds were placed in transparent polystyrene

microtiterplates (127 x 82 cm, 96 wells; Omnilabo International BV, Breda, the Netherlands), filled with tap water (one seed per well). Germination, defined as the emergence of the first foliage leaf, was checked daily for a period of 45 days.

Statistical analysis

Relationships between seed mass and seed size (width and length) were assessed by means of linear regression analysis, using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA). Differences in total seed ingestion (*i.e.* proportion of offered seeds that were ingested) and total retrieval (*i.e.* proportion of ingested seeds recovered from the faeces) were tested by mean of General Linear Modelling using the MIXED procedure for repeated measures in SAS 9.1.3 (Littell *et al.* 1998). Data were arcsine (square root) transformed to assure homoscedasticity and normality of residuals. In the analyses, seed mass and 'block' (of the RCB design) were included as fixed factors and fish individual as a random factor. Differences between seed masses (L, M and H) were tested with pairwise *post hoc* tests (with a $P<0.0167$ comparisonwise error rate, after Bonferroni correction). Variation among seed masses in retrieval rate over retention time was also analysed by means of repeated-measures ANOVA, with retention time added as a fixed factor (Charalambidou *et al.* 2003). To remove the effect of total retrieval from this analysis, data were standardized by dividing data from each retrieval event (at each measured retention time) by the total retrieval measured in that individual fish. Differences in total germination (*i.e.* proportion of seeds that germinated by the end of the germination run) were also tested using the MIXED procedure for repeated measures ANOVA (with seed mass and 'block' included as fixed factors and fish individual as a random effect), followed by pairwise *post hoc* tests comparing the germination after different treatments (fish-ingested *vs* controls) for each seed mass (with a $P<0.0167$ comparisonwise error rate, after Bonferroni correction). Differences in germination rates (*i.e.* the number of days to germination) were tested in a survival analysis by fitting a Cox proportional hazards regression to the number of days between setting for germination and seedling emergence for each individual seed that germinated, using S-plus 2000 (Mathsoft Engineering & Education Inc., Zoetermeer, the Netherlands). To separate the effects of germination rate from those of total germination, non-germinated seeds were excluded from the analysis. In addition, we fitted separate models for each seed mass category, comparing the germination rate of fish-ingested vs control seeds, with seed treatment (fish-ingested vs controls) as a fixed factor and individual as a random (or frailty) effect.

Results

The average (SE) seed mass was 14.09 (0.06) mg (N = 6463 seeds, range = 2.0 – 35.8 mg, Fig. 1). Seed mass was positively related to seed length (Linear regression: $R^2=0.159$; $P<0.0001$), seed width ($R^2=0.508$; $P<0.0001$) and a multiplication of seed length x width ($R^2=0.579$; $P<0.0001$; N = 693 seeds).

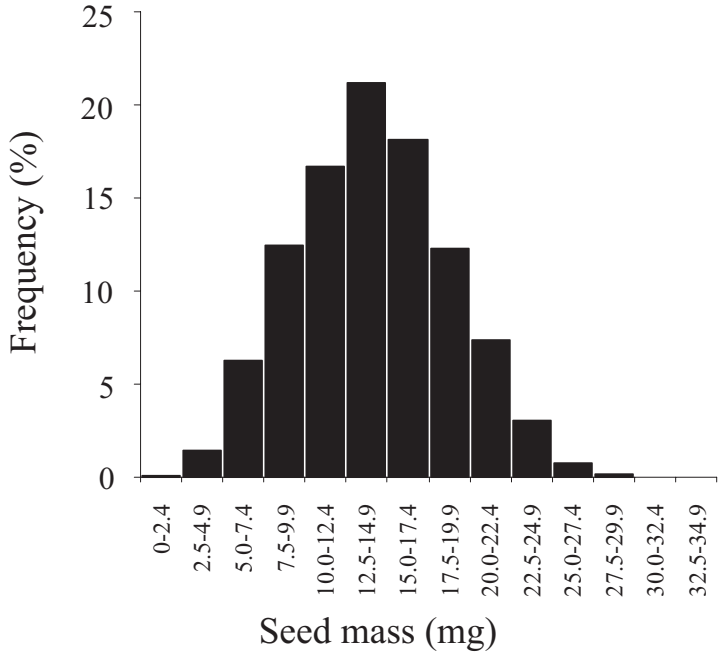

Fig. 1 Distribution of seed mass classes, based on 6462 seeds taken from 75 plants collected in 3 natural populations in the Netherlands.

The probability of ingestion of *S. emersum* seeds was significantly affected by their seed mass (RMANOVA: $F_{2,18}$=28.09, P<0.0001), with heavier seeds having a significantly lower probability of being ingested compared to lighter seeds (Fig. 2a; H vs L: P<0.0001; H vs M: P=0.0047; M vs L: P=0.0005). The factor 'block' (of the RCB-design) had no affect on the ingestion of seeds ($F_{2,9}$=0.84, P=0.4615), indicating that the order in which the seed masses were partitioned among the three repeated feeding trials did not affect their probability of ingestion.

The total retrieval of *S. emersum* seeds was also significantly affected by seed mass ($F_{2,18}$=15.44, P=0.0001), with heavier seeds having a significantly higher probability of retrieval compared to lighter seeds (Fig.. 2b; H vs L: P<0.0001; H vs M: P=0.0007; M vs L: P=0.0044). As the fish faeces contained many seed fragments, especially during the first 10 hours, the rest of the ingested seeds were most likely digested in the fish's digestive tracts. The factor block did not affect the total retrieval of seeds ($F_{2,9}$=1.04, P=0.3931), indicating that the order in which the seed masses were partitioned among the three repeated feeding trials did not affect the probability of seed retrieval. The pattern of seed retrieval over time followed a leptokurtic curve, did not differ between the three different seed masses (L, M, and H) and was not affected by the block design (seed mass effect: $F_{2,16}$=0.12, P=0.8854; block effect: $F_{2,8}$=0.02, P=0.9757) (Fig. 2c).

Seed germination (*i.e.* the total proportion of germinated seeds over retrieved seeds) was not affected by seed mass or the block effect (seed mass effect: $F_{2,14}$=1.54, P=0.2485; block effect: $F_{2,8}$=0.18, P=0.8416). Separate *post hoc* tests, comparing the germination of fish-ingested vs control seeds for each seed mass, showed that for light seeds germination of fish-ingested seeds was significantly higher than for control seeds (F=10.36; P=0.0092), while for medium and heavy seeds no difference was found (F=0.25, P=0.6261 and F=0.58, P=0.4606, respectively; Fig. 2d).

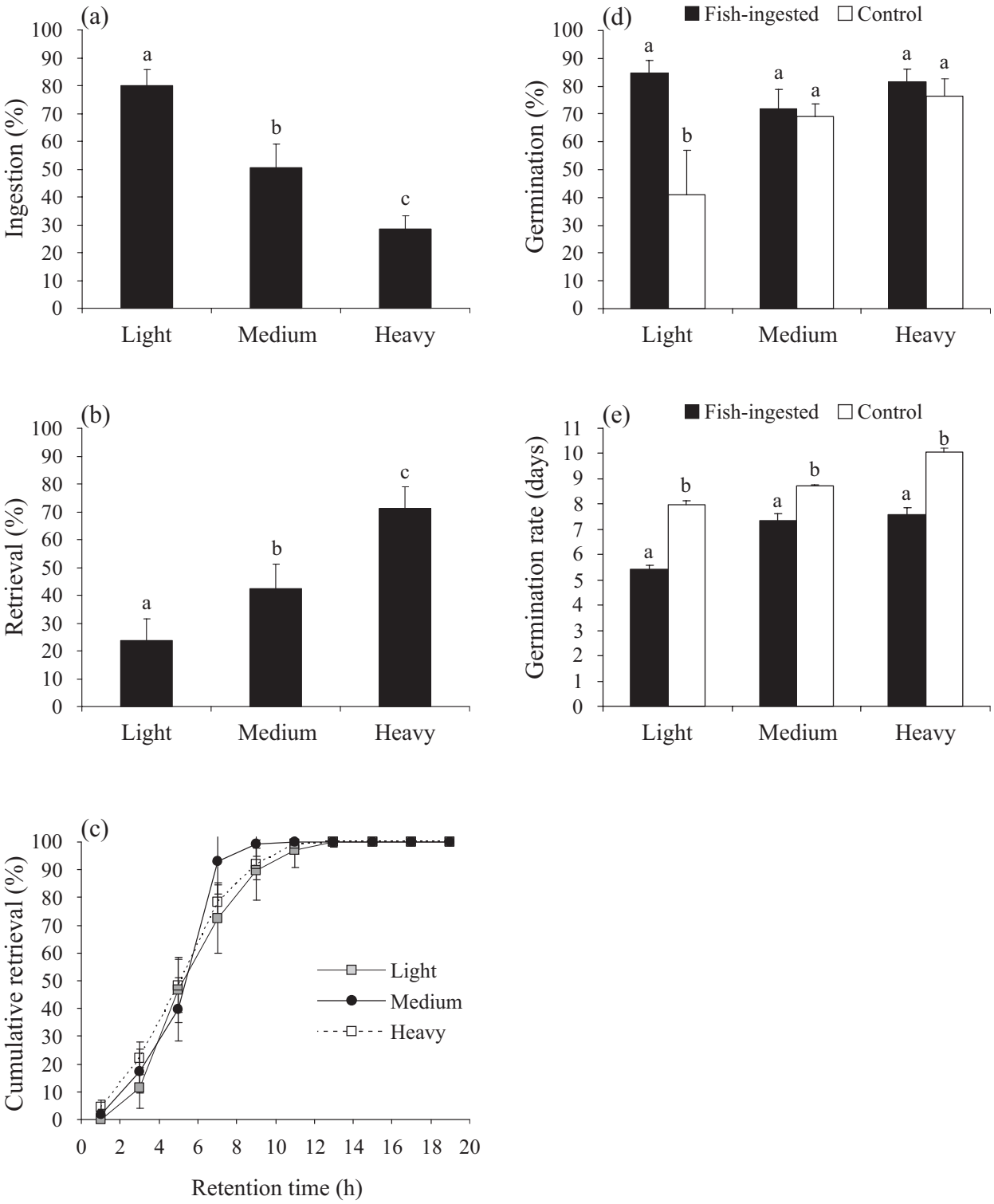

Fig. 2 Mean (± SE) **(a)** seed ingestion (%), **(b)** seed retrieval % over 24 hours, and **(c)** cumulative retrieval (%) ([number of seeds retrieved after x hours after ingestion / total number of seeds retrieved after 24 hours] x 100) of *S. emersum* seeds fed to carp (bars that do not share a common letter are significantly different from each other; see text for *P*-values). Mean (± SE) **(d)** seed germination (%) and **(e)** germination rate (number of days to germination) of fish-ingested seeds (black bars) and non-ingested control seeds (white bars; n = 150 controls for each seed mass, in three batches of 50 seeds) of *S. emersum* (adjacent bars that do not share a common letter are significantly different from each other; see text for *P*-values). The data presented in a-e is based on n = 3 repeated feeding trials, each trial with n = 12 fish (the order in which each carp was fed the L, M and H seeds in the three feeding trials, was partitioned in a randomized complete block design).

Germination rate (*i.e.* the number of days to germination) of fish-ingested seeds was significantly affected by seed mass (Cox regression: L vs M $\chi^2 = 32.39$, $P < 0.0001$; M vs H $\chi^2 = 3.05$, $P < 0.0081$), suggesting that light seeds germinated faster than heavier seeds. Separate *post hoc* tests, comparing the germination rate of fish-ingested vs control seeds, showed that for each seed mass fish-ingested seeds had a higher germination rate (*i.e.* less days to germination) than non-ingested control seeds (Cox regression: $\chi^2 = 16.2$, $P < 0.0001$; $\chi^2 = 11.1$, $P = 0.0009$; $\chi^2 = 5.24$, $P = 0.022$; for light, medium and heavy seeds, respectively; Fig. 2e).

Discussion

Some animals take up seeds unintentionally while foraging on other food sources leading to passive internal dispersal (Stiles 2000). In grassland ecosystems, for example, large herbivores may unintentionally ingest seeds while grazing (Pakeman *et al.* 2002; Mouissie *et al.* 2005; Cosyns *et al.* 2005), whereas in aquatic ecosystems, waterfowl (Figuerola *et al.* 2002; Clausen *et al.* 2002) and fish (Agami and Waisel 1988; Chick *et al.* 2003; Nurminen *et al.* 2003) may unintentionally take up seeds while foraging on vegetative plant parts or while sifting through the detritus layers on the bottom. Although unintentional, it has been suggested that in aquatic habitats that are characterized by the presence of a large number of fish and waterfowl, these animals may, collectively, constitute an important part in the dispersal of aquatic plants (Charalambidou *et al.* 2003; Pollux *et al.* 2006). The effectiveness of these animals as seed dispersers, however, depends on their probability of seed ingestion, the retention time of seeds in their digestive tracts, the probability that seeds survive a passage through their digestive tracts, and the viability and germination rate of seeds after passing through their digestive tracts.

Differences in dispersal probability between seed sizes

In fish the unintentional uptake of seeds into the oral cavity together with other food items does not preclude seed selection before actual ingestion (*i.e.* transfer from the oral cavity to the digestive tract). Fishes have complex mechanical and chemical senses for the examination of potential food items that have been taken up (Sibbing *et al.* 1986; Sibbing 1988), and unpalatable items (*e.g.* detritus, sand, stones) may be expelled by 'spitting' (a reversed suction pump action of the orobuccal and opercular cavities (Sibbing *et al.* 1986; Callan & Sanderson 2003). This selection may lead to differences in seed ingestion rates between plant species, depending on their seed characteristics. For example, softer seeded species have been shown to have a higher probability of ingestion compared to harder seeded species during ingestion by carp (Pollux *et al.* 2006). The results of this study show that heavy (larger) seeds of *S. emersum* are more likely to be identified as unpalatable items and expelled by carp than light (smaller) seeds. In nature, the probability of ingestion also depends on the relative availability of seeds (which may vary among seed sizes; Fig.. 1), however, for the purposes of this study we ensured an equal availability during our feeding experiments in order to preclude frequency-dependent seed size selection during ingestion (Celis-Diez *et al.* 2004; Bruun and Poschlod 2006).

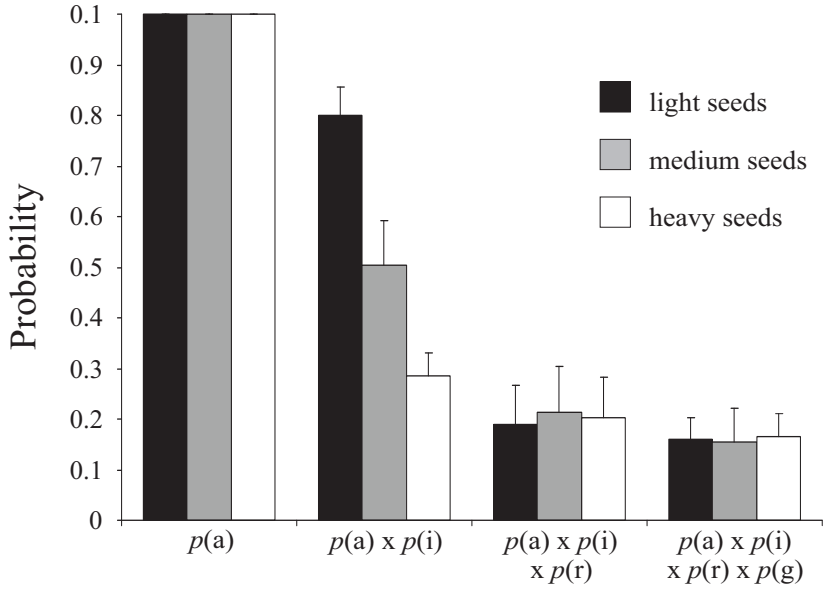

Fig. 3 A comparison of the dispersal probability between light (white bars), medium (grey bars) and heavy (black bars) seeds of *Sparganium emersum* dispersed by fish. The comparison is based on parameters inferred from the feeding experiments: *p*(a) = probability of seed availability [in this study *p*(a) is equal to 1], *p*(i) = probability of ingestion, *p*(s) probability of survival during gut passage and *p*(g) = probability of germination after gut passage.

In contrast, this study also shows that seed size has an opposite effect on seed survival during gut passage, since smaller seeds had a lower probability of retrieval compared to larger seeds. Since seed survival in the digestive tract largely depends on protection by the seed coat (Gardener *et al.* 1993a; Traveset 1998), the differences in survival rate are most likely related to differences in absolute seed coat thickness; *i.e.* larger *S. emersum* seeds have thicker seed coats compared to smaller seeds, leading to higher survival rates.

Moreover, in our study we found no significant difference between small, medium and large *S. emersum* seeds in the proportion of seeds that germinated after passing through the digestive tract of carp. However, comparisons between seeds that passed through the digestive tract of fish and non-ingested (control) seeds showed that gut passage may (slightly) enhance the germination percentage of *S. emersum* seeds ingested by carp, although the increase was not always significant. Studies have shown that a passage through the digestive tract of waterfowl (Santamaría *et al.* 2002; Pollux *et al.* 2005) or fish (Agami and Waisel 1988; Smits *et al.* 1989; Pollux *et al.* 2006) may enhance the germination percentage of species with a hard seed coat, such as *S. emersum*, due to mechanical and chemical abrasion of the seed coat leading to the breaking of seed coat dormancy (Traveset 1998; Santamaría *et al.* 2002; Pollux *et al.* 2005).

The probabilities of ingestion *p*(i), seed survival during gut passage *p*(s) and germination after gut passage *p*(g), inferred from our study, may be used to compare the overall dispersal probabilities *p*(d) between the different seed size categories, by calculating the dispersal probability for each seed size as follows: *p*(d) = *p*(a) x *p*(i) x *p*(s) x *p*(g) (Pollux *et al.* 2006). Note that in our feeding experiments, the availability of the seeds was kept equal [*p*(a) = 1] to ensure that seed size selection

during ingestion was not frequency-dependent (Celis-Diez *et al.* 2004; Fig.. 3). The results of this study suggest that there are no significant differences in the dispersal probability of differently sized *S. emersum* seeds, because the initial reduction in probability of ingestion *p*(i) with increasing seed size (L = 0.7989, M = 0.5044 and H = 0.2858, respectively) is counterbalanced by an increased probability of seed survival *p*(s) (L = 0.2364, M = 0.4224 and H = 0.7103), while the probability of germination of retrieved seeds p(g) remains unaffected by seed size (ranging 0.7201-0.8488; Fig.. 3).

Differences in potential dispersal distance between seed sizes

Several studies have shown that the size of a seed may determine the time it remains in an animal's digestive tract (Gardener *et al.* 1993a; Traveset 1998), often with large and heavy seeds having shorter retention times in the digestive tract of vertebrates (*e.g.* birds, cattle, primates) than small and light seeds, potentially leading to shorter dispersal distances (Traveset 1998, and references therein). However, this study did not reveal a significant effect of seed size of *S. emersum* seeds on the seed retention times in the digestive tract of carp. These findings are in accordance with other studies that show that, in fish, the size, hardness and biochemical composition of food items (*e.g.* seeds, invertebrates, prey fish) have little effect on the gastric evacuation rate (*i.e.* the time required to evacuate the stomach content; Pollux *et al.* 2006; and references therein). It is therefore suggested that seed size affects the retention time of seeds passing through the digestive tracts of vertebrates with highly specialized digestive systems (*e.g.* birds, mammals), but not of seeds passing through the relatively unspecialized digestive tracts of fish (Traveset 1998; Pollux *et al.* 2006).

Differences in potential competitive (dis)advantages between seed sizes

Ultimately, the success of a dispersal event also depends on the vigour of the seedling once it has been deposited in a new habitat. Early seedling emergence may either result in competitive advantages (*e.g.* longer growth season) or competitive disadvantages over later emerging seedlings (*e.g.* increased risk of seedling mortality due to predation or unpredictable harsh weather conditions), depending both on the plant species and the prevailing environmental conditions at the time of establishment (Traveset 1998; Verdú and Traveset 2005).

Our experiments revealed a significant faster germination rate (*i.e.* the number of days to germination) of smaller compared to larger fish-ingested *S. emersum* seeds (with differences in germination rate ranging from 1 to 3 days). These differences are most likely related to differences in seed coat thickness. The thinner seed coats of small *S. emersum* seeds may have sustained relatively more damage (*i.e.* a higher level of abrasion) during gut passage compared to the thicker seed coats of larger seeds. Since abrasion during gut passage may enhance germination (Traveset 1998), and may have resulted in higher germination rates for smaller compared to larger *S. emersum* seeds. The results, furthermore, showed that, for each seed size, fish-ingested seeds had a faster germination rate as non-ingested (control) seeds, again most likely due to abrasion of the seed coat during gut

passage (Traveset 1998; Traveset *et al.* 2001; Santamaría *et al.* 2002; Pollux *et al.* 2005).

However, it has been argued (i) that such short time advantages during early life stages may have little effect on later plant performance (Verdú and Traveset 2005), particularly in aquatic environments (Figuerola & Green 2004; Figuerola *et al.* 2005), and (ii) that observed benefits of earlier emergence on plant growth during early life stages, found under optimal controlled experimental conditions, may be absent under field conditions because, here, a multitude of environmental factors may negatively affect the growth of early seedlings effectively reducing differences between early and late seedlings (Verdú and Traveset 2005). It might therefore be argued, that the small differences in germination rate between differently sized seeds of *S. emersum* might be too small to be translated into competitive (dis)advantages among conspecific seedlings under natural conditions in the field.

General conclusions

In conclusion, the results of this study suggest that there are no, or very little, differences in: (i) the probability of dispersal between differently sized *S. emersum* seeds (assuming an equal availability of seeds), because the negative effect of increased seed size on the probability of seed ingestion by carp is counterbalanced by a positive effect on the probability of seed survival during gut passage, while the probability of germination after gut passage remains unaffected; (ii) the dispersal distance of differently sized *S. emersum* seeds, because the time that seeds remain in the digestive tracts of the fish is not affected by their seed size; and (iii) the post-establishment growth and competitive ability of seedlings of differently sized *S. emersum* seeds, because the differences in germination rate obtained under controlled conditions were, although significant, so small that, arguably, no effect on later plant performance is expected under field conditions. Finally, this study shows the importance of studying all successive stages of the endozoochorous dispersal process when comparing dispersal probabilities within and between plant species, because seeds with different phenotypic traits (*e.g.* size, structure, morphology, etc.) may have different (potentially conflicting) effects on each of these stages, ultimately affecting probability of dispersal, dispersal distance on seedling emergence.

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Chapter 3

The effect of seed morphology on the potential dispersal of aquatic macrophytes by the common carp (*Cyprinus carpio*)

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Summary

1. The potential for seed dispersal by fish (ichthyochory) will vary among aquatic plants due to differences in seed size and morphology.

2. To examine how seed morphology influences the probability of dispersal by the common carp (Cyprinus carpio), we studied seed ingestion, retention time, egestion and germination of seeds of Sparganium emersum and Sagittaria sagittifolia, two aquatic plant species with similar sized but morphologically different seeds.

3. We compared dispersal probabilities between the two plant species, in which the probability of dispersal is assumed to be a function of the probabilities of seed ingestion, retrieval and germination, and the dispersal distance is assumed to be a function of seed retrieval rate (through excretion) over time.

4. We found that, although the soft seeds of Sagittaria sagittifolia had an approximately 1.5 times higher probability of being ingested by the carp than the hard seeds of Sparganium emersum (83.15 ± 1.8% vs 56.16 ± 2.7%, respectively), the latter had an almost two-fold higher probability of surviving the passage through the digestive tract (38.58 ± 2.7% vs 20.97 ± 1.5%, resp.). Patterns of seed egestion over time did not differ between the two plant species, despite the difference in seed morphology. Gut passage had a different effect on seed germination between plant species. Compared to non-ingested controls, seeds of Sparganium emersum showed a 12.6 % increase in germination and a 2.1 day acceleration in germination rate, whereas seeds of Sagittaria sagittifolia displayed a 47.3 % decrease and 5.1 day delay, respectively.

5. Our results suggest that seed morphology affects the dispersal probability and post-dispersal establishment, but not the dispersal distance, of aquatic plants that are dispersed by fish.

Introduction

Animal-assisted transport of progagules has long been considered an important mode of plant dispersal in aquatic environments (Darwin, 1859; Ridley, 1930). Waterbirds and fish are among the most likely candidates to play a role in the zoochorous dispersal of aquatic plants (Cook, 1988; Barrat-Segretain, 1996). While seed dispersal by waterfowl has received considerable attention (*e.g.* Figuerola, Green & Santamaría, 2002; Clausen *et al.,* 2002; Charalambidou, Santamaría & Langevoord, 2003; Charalambidou *et al.*, 2005; Pollux, Santamaría & Ouborg, 2005), seed dispersal by fish has not been studied systematically. Though circumstantial, there are a number of findings that indicate that seed dispersal by fish (*i.e.* ichthyochory) may be important. Firstly, stomach-content analyses on temperate European and North American fishes show the presence of seeds in many different species (Ridley, 1930; Crivelli, 1981; Bergers, 1991; García-Berthou, 2001; Nurminen *et al.,* 2003; Chick, Cosgriff & Gittinger, 2003; van Riel, unpublished). Furthermore, on average, 1-5 % of the field-collected individuals of these species bear seeds in their stomachs (Bergers, 1991; van Riel, unpublished). Occasionally, seeds are found in a much larger proportion of the fish population; *e.g.* 73-78 % of the channel catfish (*Ictalurus punctatus*, Rafinesque) from the Mississippi River (USA), and 42 to 93 % of the common carp (*Cyprinus carpio*, L.) from Lake Banyoles and the Carmargue (Spain and France, respectively) (Crivelli, 1981; García-Berthou, 2001; Chick *et al.,* 2003). Moreover, observed seed quantities in the stomachs of individual fish range from a few to more than a 1000 seeds per stomach (Ridley, 1930; Crivelli, 1981; Bergers, 1991; Nurminen *et al.*, 2003; Chick *et al.,* 2003). Combining the prevalence of seeds in fish stomachs with the fact that many lake and river systems may harbour high numbers of fish (easily reaching several hundreds of thousands; *e.g.* van Densen, Steinmetz & Hughes, 1990), suggests that, collectively, fish may play an important role in the dispersal of temperate and aquatic riparian plants.

However, little is known about the role of seed morphology on seed ingestion during fish feeding, although this factor may determine which plant species are actually dispersed. Furthermore, from studies using various terrestrial animal models we know that passage rates, the proportion of seeds egested, and the germination potential of ingested seeds are influenced by the size and morphology of the seeds (Traveset, 1998), although this has rarely been investigated in fish (Agami & Waisel, 1988; Smits, van Ruremonde & van der Velde, 1989; Traveset, 1998). The purpose of this study was to determine whether aquatic plants with different seed structures differ in their potential for ichthyochoric dispersal. Using common carp (*Cyprinus carpio*) we compared the ingestion, retention time, and subsequent egestion and germination of seeds of unbranched bur-reed (*Sparganium emersum* Rehmann 1872, Sparganiaceae) and arrowhead (*Sagittaria sagittifolia* Linnaeus 1753, Alismataceae). We used a modelling approach for comparing dispersal probabilities between the two plant species, in which (i) the probability of dispersal is assumed to be a function of the probabilities of seed ingestion, retrieval and germination, and (ii) the dispersal distance is assumed to be a function of the retrieval rate over time. We hypothesized that *S. emersum* would show a lower probability of ingestion, but a higher probability of retrieval and germinability, owing to the hard scleridial seed coat, as compared with the soft seed coat of *S. sagittifolia*.

Material & Methods

Study species

The common carp *Cyprinus carpio* is one of the most widely spread freshwater fish species, commonly found in lakes, canals and lowland rivers in temperate and tropical regions of Eurasia and North America. Dietary studies on field-collected individuals have shown that *C. carpio* is an opportunistic omnivorous forager that includes macrophyte seeds in its diet (Ridley, 1930; Crivelli, 1981; Bergers, 1991; García-Berthou, 2001). *Sparganium emersum* and *Sagittaria sagittifolia* are helophyte plant species that are also widely distributed along canals and lowland streams throughout Eurasia and North America (Cook & Nicholls, 1986). The seeds of the two species are similar in size, but differ greatly in their morphology. The drupe-like fruit of *S. emersum* consists of a seed enclosed in a hard scleridial endocarp and a tough spongy mesocarp, with a plugged pointy micropyle (Cook & Nicholls, 1986). The fruit of *S. sagittifolia* consists of a nutlet-like seed surrounded by a soft membranous endocarp and a fleshy, semi-transparent, laterally compressed disc-like mesocarp. The common carp (*C. carpio*) and the two plant species (*S. emersum* and *S. sagittifolia*) overlap in their distribution, and it has been suggested that seeds of both plant species may be dispersed by carp (Hochreutiner, 1899; reference taken from Ridley, 1930).

Experimental design

Ripe seeds of *S. emersum* and *S. sagittifolia* were collected during October 2003 from natural populations in the Netherlands. The seeds of both species need to be cold-stratified (*i.e.* subjected to cold temperature for an extended period) while being immersed in water, to break seed dormancy (Muenscher, 1936). Therefore, the seeds were stored in glass jars filled with tap water, in a dark cold room at 5 ± 1 ºC, to mimic natural conditions of Central-North European winters.

Twelve common carp with a mean mass of 0.307 ± 0.01 (SE) kg were obtained from Ruud Vonk Fish Hatchery (Maurik, the Netherlands) in October 2003. The fish were individually kept in 100-L tanks in the fish facilities of Radboud University Nijmegen, the Netherlands and fed daily on a fixed diet of commercial pellets (Trouvit, Trouw & Co, Putten, the Netherlands) amounting to 1% of their body mass. The water in the tanks was maintained at 24ºC and was continuously aerated and refreshed (50 L h^{-1}). To ensure homogenisation of water quality among the twelve tanks, all were supplied with water coming from the same filtering system.

From January to April 2004, we performed 12 feeding trials at weekly intervals. At the beginning of a feeding trial, each of the twelve fish was fed a total of 10 Trouvit food pellets (each pellet containing five randomly selected *S. emersum* and five *S. sagittifolia* seeds). Five to ten minutes after feeding, non-ingested seeds (*i.e.* seeds that were expelled by 'spitting'; Sibbing, Osse & Terlouw, 1986) were removed from the tanks with aquarium nets (frame size 10x15 cm; mesh size 1 mm) and counted. Fish faeces were then collected every 2 hours from the bottom of the tanks by means of aquarium nets for a period of 24 hours (preliminary tests, lasting 48 hours, showed that the fish always egested all non-digested seeds well within 24 hours). Collected faeces

were immediately rinsed with tap water and sieved using a 500 μm square mesh size sieve (diameter 19 cm). Seeds retrieved were transferred to plastic containers (100 ml) filled with tap water and returned to the dark cold room (5 ± 1 ºC) for the remainder of the experiment to ensure an equal cold-stratification period for all seeds in all feeding trials (from seed collection in the field in October 2003 to the germination test in May 2004). For each plant species, three batches of 50 randomly selected non-ingested seeds, were used as controls in the germination experiment. These control seeds received a similar pre- and post-experimental treatment as the seeds used in the feeding experiments (*i.e.* placed in soft pellets soaked in water, sieved with tap water and stored at 5 ± 1 ºC for the remainder of the feeding experiments) to exclude possible effects of pre- or post-feeding treatment of the seeds.

In May 2004, all the retrieved and control seeds were set to germinate simultaneously in a climate chamber with a photoperiod of 16L/8D, a daytime irradiance of 200 μmol photons s^{-1} m^{-2} and a day/night temperature cycle of 25/18 ºC. Seeds were placed in transparent polystyrene microtiterplates (127 x 82 cm, 96 wells; Omnilabo International BV, Breda, the Netherlands), filled with tap water (one seed per well). Germination, defined as the emergence of the first foliage leaf, was checked daily for a period of 45 days.

Statistical analysis

Differences in total seed ingestion (*i.e.* proportion of offered seeds that were ingested) and total retrieval (*i.e.* proportion of ingested seeds recovered from the faeces) were tested by means of General Linear Modelling using the MIXED module for repeated measures in SAS 9.1.2 (Littell, Henry & Ammerman, 1998; SAS Institute Inc, Cary, NC, USA). Data were arcsine transformed to assure homoscedasticity and the normality of residuals. In the analyses, plant species was added as a fixed factor and feeding trial and fish individual as random factors. Variation between plant species in seed retrieval over retention time was also analysed by means of repeated-measures ANOVA, with retention time added as an additional fixed factor (Charalambidou *et al.,* 2003). To remove the effect of total retrieval from this analysis, data were standardized by dividing data from each retrieval event (*i.e.* as measured at each retention time interval) by the total retrieval measured in that individual fish. The effect of plant species and seed treatment (*i.e.* control vs fish-ingested) on total germination (*i.e.* proportion of seeds that germinated by the end of the germination run), were tested using repeated-measures ANOVA, with plant species added as a fixed factor and feeding trial and fish individual as random factors, followed by pairwise *post hoc* tests comparing the different treatments within each plant species (with a $P < 0.025$ comparisonwise error rate, after Bonferroni correction). Differences in germination rate were tested in a survival analysis by fitting a Cox proportional hazards regression to the number of days between setting for germination and seedling emergence, for each individual seed that germinated, using S-Plus 2000 (Mathsoft Engineering & Education Inc., Zoetermeer, the Netherlands). To separate the effects of germination rate from those of total germination, non-germinated seeds were excluded from the analysis. For each plant species we fitted separate models, with seed treatment as a fixed factor, and

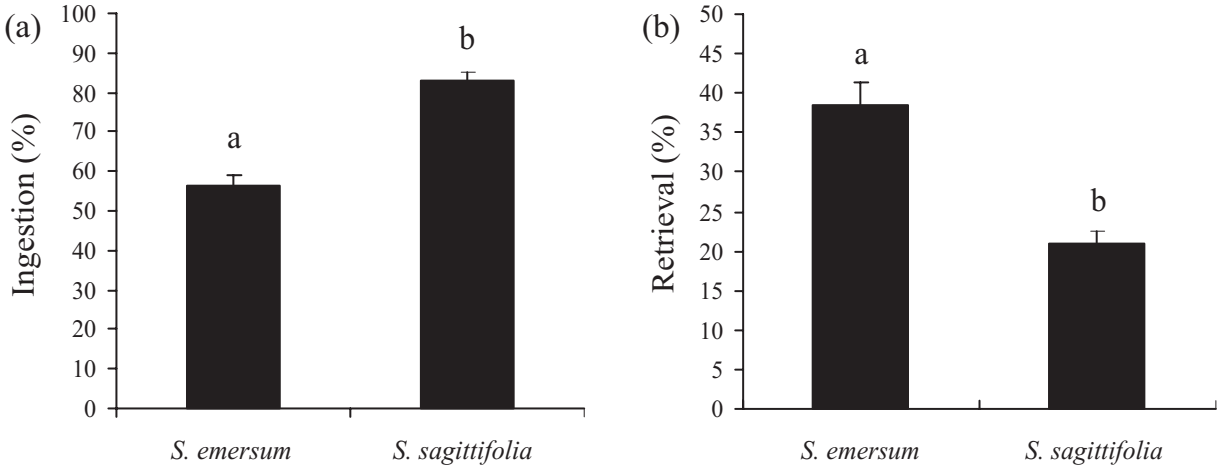

Fig. 1 (a) Mean (± SE) seed ingestion (%), and **(b)** seed retrieval (%) over 24 hours, of *S. emersum* and *S. sagittifolia* seeds fed to carp (n = 12 feeding trials, each trial with n = 12 fish). Bars that do not share a common letter are significantly different from each other (see text for *P*-values).

individual (for fish-ingested) or batch replicate (for controls), respectively, as a random (or frailty) effect.

Results

All food pellets with seeds offered to the carp were eaten, *i.e.* they were taken into the oral cavity where they were 'chewed upon' (*i.e.* process of oral examination of food; Sibbing *et al.,* 1986). Shortly afterwards, items that were apparently unpalatable were expelled by means of 'spitting'. Only seeds were expelled, the rest of the food pellets were always (re-)ingested. The results revealed a significant difference in total seed ingestion (*i.e.* the proportion of ingested seeds over seeds offered) between the two plant species ($F_{1,275}$=21.65, P<0.0001). The hard, pointed seeds of *S. emersum* had a significantly lower ingestion of 56.16 ± 2.7 (SE) %, compared with the softer *S. sagittifolia* seeds with 83.15 ± 1.8 % being ingested (Fig.. 1a).

The total retrieval (*i.e.* the proportion of retrieved seeds after egestion over seeds ingested) differed significantly between the two plant species ($F_{1,268}$=13.44, P=0.0003), being higher for *S. emersum* (38.58 ± 2.7 %) than in *S. sagittifolia* (20.97 ± 1.5 %; Fig. 1b). As the fish faeces contained many seed fragments, especially during the first 10 hours, the rest of the ingested seeds were probably digested. The pattern of seed retrieval over time followed a leptokurtic curve which was indistinguishable for the two plant species ($F_{1,11}$<0.001, P=0.9979; Fig. 2). For both species, maximum seed retrieval was observed at 8 hours, and the last seeds were found in the faeces after 18 (for *S. sagittifolia*) to 20 (*S. emersum*) hours after ingestion.

Seed germination (*i.e.* the total proportion of germinated seeds over retrieved seeds) was higher for *S. emersum* than for *S. sagittifolia* ($F_{1,226}$=461.24, P<0.0001; Fig.. 3a). For *S. emersum*, germination of non-ingested control seeds (70.67 ± 4.1 %) did not differ significantly from fish-ingested seeds (83.27 ± 2.2 %; F_1 = 1.30, P = 0.2569), while for *S. sagittifolia*, control seeds showed

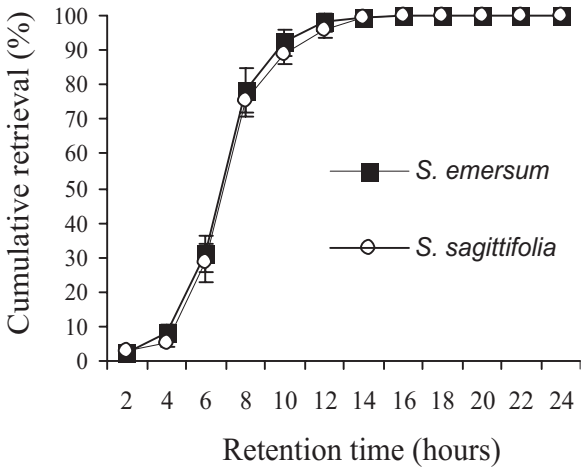

Fig. 2 Mean (± SE) cumulative retrieval (%) ([number of seeds egested after x hours after ingestion/total number of seeds egested] x 100) for *S. emersum* and *S. sagittifolia* seeds ingested by carp (n = 12 feeding trials, each trial with n = 12 fish).

a significantly higher total germination than fish-ingested seeds (72.33 ± 3.8 % and 25.04 ± 2.3 %, respectively; F_1 = 6.54, P = 0.012; Fig.. 3a). Control seeds of *S. emersum* did, however, display a slower germination rate (*i.e.* number of days to germination) compared to fish-ingested seeds (Cox regression: χ^2=39.1, df=1, P<0.001), as opposed to *S. sagittifolia*, where control seeds displayed significantly faster germination rates compared to fish-ingested seeds (Cox regression: χ^2 =36.4, df=1, P<0.001; Fig. 3b).

Discussion

Ingestion

Analyses of the stomach contents of fish caught in the field, show that temperate species, particularly cyprinids such as *C. carpio*, ingest seeds as part of their diet (Crivelli, 1981; Bergers, 1991; García-Berthou, 2001; Nurminen *et al.,* 2003). Little is known about which plant species are ingested, however, or whether certain seed structures might affect the probability of ingestion. This study shows that under controlled conditions the soft seeds of *S. sagittifolia* are 1.5 times more likely to be ingested than the hard seeds of *S. emersum*. Several studies have reported highly complex food selection mechanisms in temperate cyprinids, involving morphological and behavioral adaptations as well as mechanical and chemical senses, for the detection and investigation of potential food items (Sibbing *et al.,* 1986; Sibbing, 1988; Callan & Sanderson, 2003). For many temperate cyprinids these mechanisms are crucial since they take up their food along with unpalatable debris (*e.g.* detritus, sand, stones). In the oral cavity, palatable and unpalatable items are separated, and the unpalatable particles expelled by 'spitting' (a reversed suction pump action of the orobuccal and opercular cavities; Sibbing *et al.,* 1986; Callan & Sanderson, 2003). Our results show that the hard, pointed, drupe-like seeds of *S. emersum* are more likely to be identified as unpalatable items and expelled by

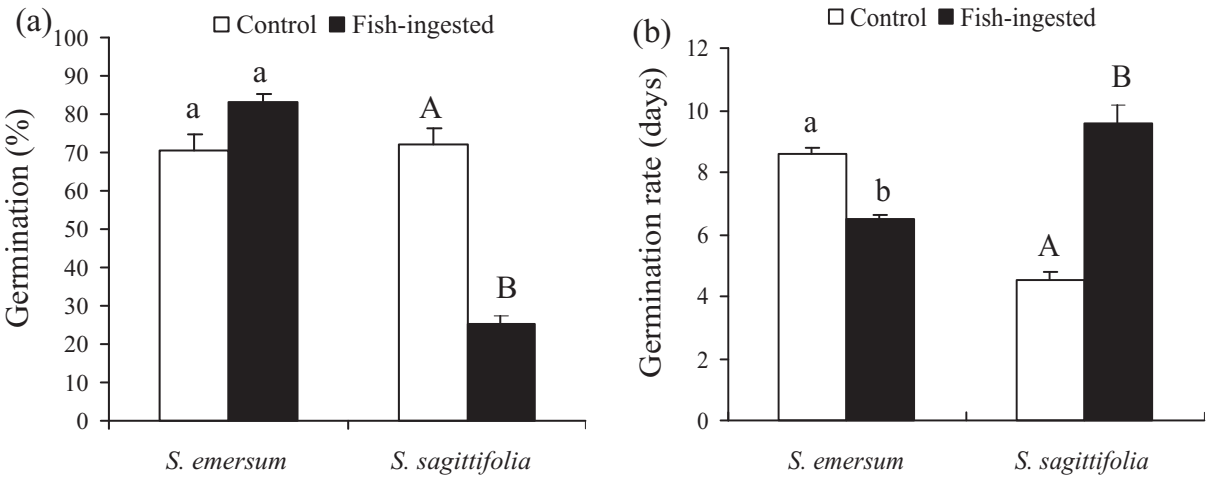

Fig. 3 (a) Mean (± SE) seed germination (%), and **(b)** germination rate (number of days to germination) of non-ingested control (n = 150, in three batches of 50 seeds) and fish-ingested (n = 12 feeding trials, each trial with n = 12 fish) seeds of *S. emersum* and *S. sagittifolia*. For each species, significant differences between control and fish-ingested seeds were indicated with letters (a and b for *S. emersum* and A and B for *S. sagittifolia*). Bars that do not share a common letter are significantly different from each other (see text for *P*-values).

carp than the soft, fleshy, disc-like seeds of *S. sagittifolia*, and thus that seed characteristics are likely to affect seed ingestion by fishes.

Retention time and total retrieval

We found no difference in seed retention time in the digestive tract of carp between *S. emersum* and *S. sagittifolia*. However, this outcome is consistent with studies examining the effect of food type on the gastric evacuation rate of fish (*i.e.* the time required to evacuate the stomach content). These studies used a wide variety of invertebrate prey species, often with large differences in size, carapace hardiness and biochemical composition, and yet reported little difference in the evacuation rates (Persson, 1979; Persson, 1982; Brodeur, 1984; Nilsson & Brönmark, 2000). Therefore, we suggest that in animals with a relatively unspecialized gut morphology, such as fish, seed morphology does not affect seed retention time (this study), as opposed to animals with a highly specialized gut morphology, such as waterfowl, where seed morphology significantly affects seed retention times (Pollux *et al.*, 2005).

Furthermore, seed survival during gut passage is known to depend on a complex interaction between the characteristics of the seeds and of the animal consumers (Traveset, 1998; Charalambidou & Santamaría, 2002). For instance, in large mammalian herbivores (sheep, cattle, horses) small-seeded species tend to survive better than large-seeded species, probably because the latter sustain more mechanical damage by chewing (Pakeman, Digneffe & Small, 2002; Mouissie *et al.,* 2005). However, in animals that lack this initial mechanical chewing stage, *e.g.* waterfowl, the hardness of the seed coat appears to be more important than seed size (Proctor, 1968; Charalambidou &

Santamaría, 2002; Pollux *et al.,* 2005). Our study suggests that also in fish, seed coat hardiness is an important factor for seed survival, with the harder seeds of *S. emersum* having an almost two-fold higher probability of retrieval compared to *S. sagittifolia*. This concurs with two studies by Smits *et al.* (1989), who showed that seeds of three nymphaeid waterplants had a lower probability of being egested intact compared to seeds of two (harder-seeded) *Potamogeton* species when fed to carp, and by Agami & Waisel (1988) who retrieved a greater percentage of hard seeds than of the soft seeds of *Najas marina* after ingestion by fish.

Seed viability

Passage through the guts of vertebrate frugivores may affect seed germination (either positively or negatively) by: (i) removal of the fruit's pulp or the germination inhibitors within it, and (ii) the mechanical/chemical treatment of the seed coat in the animal's gut (Traveset, 1998). Our study revealed a decreased germination for *S. sagittifolia* and an increased germination for *S. emersum* seeds that passed through the intestinal tract of carp. The reduction in germination and germination rate of the soft *S. sagittifolia* seeds is most likely to be due to the bruising of the seed embryo reducing its capacity to germinate. The increase in germination and germination rate of *S. emersum* seeds is probably related to the breaking of the seed coat dormancy by mechanical abrasion or removal of the seed coat (Baskin & Baskin, 1998), which is necessary before the seeds can germinate (Cook, 1962). Under natural conditions the seed coat dormancy of *S. emersum* can be broken after a period of freezing, or by natural decomposition. Alternatively, seed coat dormancy of hard coated seeds of aquatic plants may be broken by passage through the digestive tract of fishes (Agami & Waisel, 1988; Smits *et al.,* 1989; this study) and waterfowl (Santamaría *et al.,* 2002; Pollux *et al.*, 2005), leading to increased germination.

A simple model for comparing dispersal probabilities between plant species

Seed dispersal by animals is often studied by means of seed feeding-experiments. These studies are designed to estimate a number of parameters, which are used to predict the probability and distance of dispersal. These are (i) the probability of seed ingestion p(i), which yields information on feeding preferences or food selection mechanisms, (ii) the probability of seed retrieval p(r), which yields information about the survival of seeds during gut passage, (iii) seed retention time, which (in combination with information on migration patterns of the animal disperser) yields information about the dispersal curve and potential dispersal distances, (iv) the probability of seed germination p(g) of retrieved seeds, which yields information about the probability of seed establishment after gut passage and, (v) germination rate, which yields information about competitive (dis)advantages over non-ingested conspecific seeds, that may arise from an earlier or later onset of germination.

In combination with the relative availability of seeds p(a), this kind of information can be used to compare the dispersal probability of different plant and animal species. In the field the availability of seeds may vary widely, both within species (between different locations) and between species,

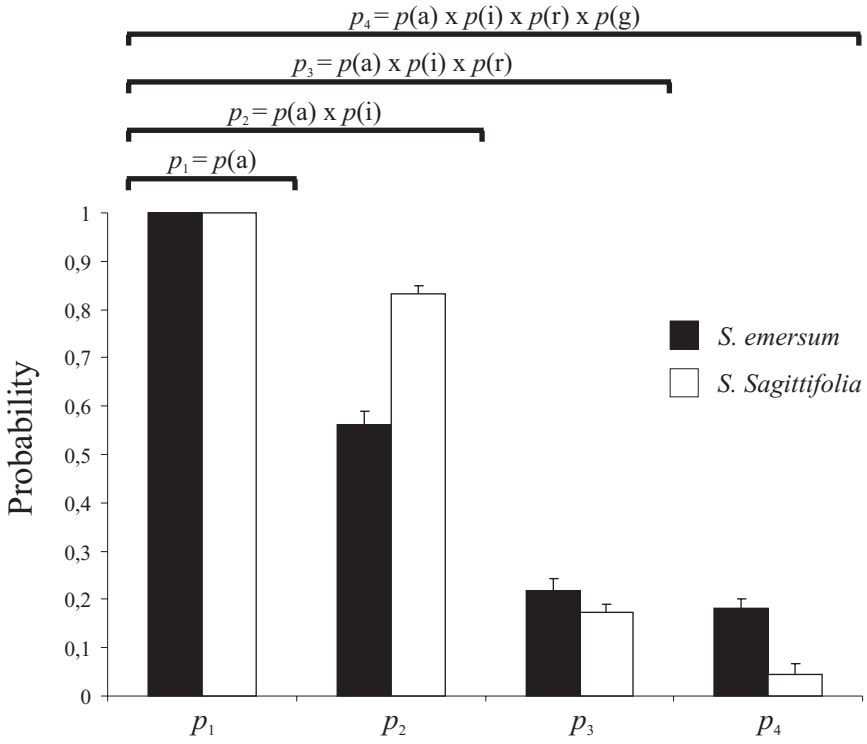

Fig. 4 A mechanistic approach to comparing the probability of fish-mediated dispersal of *S. emersum* and *S. sagittifolia*, as derived from four successive steps (see text for explanation). The comparison is based on parameters inferred from the feeding experiments: p(a) = probability of seed availability (in this study p(a) is equal to 1), p(i) = probability of ingestion, p(r) = probability of egestion, and p(g) = probability of germination.

largely depending on the distribution of the plants and their reproductive output (which in turn may both vary widely across environmental conditions). For the purposes of this study, however, we ensured an equal availability of seeds of both plant species in the food pellets (p(a)=1). Under the assumption that both plant species have a similar seed availability to the fish (p(a)=1), our results show that, although *S. sagittifolia* has a 1.5 times higher probability of being ingested, this initial advantage is nullified by a two-fold higher probability of seed egestion in *S. emersum* combined with a three times higher probability of seed germination. The results thus suggest that *S. emersum* has a higher probability (calculated as $p_4 = p(a) \times p(i) \times p(r) \times p(g)$, see Fig 4) of being dispersed by carp, compared to *S. sagittifolia* (p = 0.1804 and 0.0437, respectively). Since, there are no significant differences between both plant species in their seed retention times, differences in dispersal distances arising from carp-mediated dispersal would not be predicted. Finally, although there are clear contrasting effects on germination rate (of egested compared to control seeds) between the two plant species (with an increase for *S. emersum* and decrease for *S. sagittifolia*), a recent study by Figuerola *et al.,* (2005) has shown that such short time (dis)advantages are not likely to result in de- or increased plant performances over longer time periods. Thus, we must conclude that, based on all the parameters measured in this study, *S. emersum* has: (i) an overall higher potential for carp-mediated dispersal and post-dispersal establishment and (ii) an equal dispersal curve, indicating equal dispersal distances arising from carp-mediated dispersal, compared to *S. sagittifolia*.

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Chapter 4

Differences in endozoochorous dispersal between aquatic plant species, with reference to plant population persistence in rivers

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Summary

1. In river ecosystems, populations are continuously subjected to unidirectional downstream currents resulting in a downstream movement of populations. To ensure long-term population persistence in rivers, organisms must have a mechanism for upstream dispersal, which allows them to re-colonise upstream areas.

2. In this study we assessed differences in the potential for endozoochorous seed dispersal of Sparganium emersum and Sagittaria sagittifolia, two aquatic plant species with different seed morphologies, by mallard (Anas platyrhynchos) and teal (Anas crecca), two duck species with different body weights.

3. We found no significant differences in seed retrieval (the proportion of ingested seeds retrieved after gut passage) and seed retention time (time between seed ingestion and retrieval), between mallard and teal, despite the difference in body weights. We did find a significantly higher germination (%) over retention time of S. emersum seeds retrieved from teal compared to mallard, most likely related to a more efficient removal of the seed coat during passage through the gut of teal.

4. There were large differences between S. emersum vs. S. sagittifolia in: (i) seed retrieval (22.65±20.8% vs. 1.60±2.4%, respectively); (ii) seed retention time in duck gut, with a maximum of 60 hours vs. 12 hours; (iii) the effect of gut passage on seed germination, with an increase of approximately 35% vs. a decrease of 25%; and (iv) the effect of gut passage on seed germination rate, with an acceleration of 10 days vs. a delay of 3 days on average. The results show that S. emersum has a higher potential for endozoochorous dispersal by ducks and post-dispersal establishment than S. sagittifolia.

5. We propose that in rivers, bird-mediated seed dispersal may promote re-colonization of upstream areas, enabling long-term plant population persistence.

Introduction

Rivers and streams are linear habitats that impose special constraints on dispersal and persistence of aquatic organisms (Imbert & Lefèvre, 2003). Populations in rivers are continuously subjected to water currents and, although most species display adaptations that prevent them from being washed away under normal circumstances, in the absence of a mechanism for upstream dispersal, any advection (no matter how small) will ensure that, on average, populations will move downstream, preventing long-term persistence (Speirs & Gurney, 2001). In addition, during catastrophic and aperiodic floodings whole populations may be washed away to downstream areas (Stelter *et al.*, 1997). Hence, aquatic organisms that have stable and persistent populations in river ecosystems may be expected to display mechanisms for upstream dispersal, either active or passive.

For aquatic invertebrates this problem has received considerable attention starting with Müller's (1954) concept of ´the colonization cycle´ whereby aquatic insects compensate for the gradual downstream movements of the larvae by actively flying (Müller, 1974; Speirs & Gurney, 2001), swimming or crawling upstream (Humphries & Ruxton, 2002). However, in contrast to aquatic insects, other invertebrate species may display a sessile adult life-style and therefore lack a means of active upstream dispersal. For example, the zebra mussel *Dreissena polymorpha* (Pallas, 1771) has a sessile adult life-style and short-lived, free-swimming larvae with limited swimming capabilities, leading to downstream dispersal in rivers. Despite these difficulties, during the last two centuries zebra mussels have succeeded in colonising all main European rivers due to passive (human-mediated) upstream dispersal (Kinzelbach, 1992; Pollux *et al.*, 2003). Aquatic plants in rivers face similar difficulties, *i.e.* a sessile adult life-style and passive hydrochorous downstream dispersal of propagules (seeds and plant fragments). This, together with the notion that all over the world aquatic plants can be found in riverine systems, suggests that they must also possess a means of assisted upstream dispersal. Surprisingly, the problem of upstream dispersal for ensuring plant population persistence in river ecosystems has received little attention.

Aquatic plants are generally dispersed by water (Sculthorpe, 1967; Cook, 1988) and in rivers water flow can be considered the main dispersal vector leading to dispersal in the downstream direction (Barrat-Segretain, 1996). In addition, it has been suggested that animals, particularly fishes and water birds, may play a role in the dispersal of aquatic plants (Green, Figuerola & Sanchez, 2002; Clausen *et al.*, 2002). The role of waterbirds in the passive endozoochorous dispersal of seeds was recognised a long time ago (Ridley, 1930 and references therein); however, most evidence for such dispersal was anecdotal and detailed quantitative data were lacking. Since then, few experimental studies have investigated the mechanism or frequency of endozoochorous transport by waterfowl (Figuerola & Green, 2002; Charalambidou & Santamaria, 2002). Based on the information gained to date, Charalambidou & Santamaria (2002) identified several gaps in our knowledge of waterbird dispersal. Among these, comparisons between duck species exhibiting different diets and/or body sizes, and comparisons between plant species with different seed characteristics (*e.g.* size and morphology) were considered to be of particular interest.

In this study we assessed whether ducks might function as dispersal agents for seeds of two aquatic plant species with different seed morphologies but comparable habitat requirements,

unbranched burr-reed *Sparganium emersum* (Rehmann, 1872; Sparganiaceae) and arrowhead *Sagittaria sagittifolia* (Linnaeus, 1753; Alismataceae). In particular, we were interested in whether the two plant species differ in their potential for endozoochorous dispersal by waterfowl, and if these differences could be related to their distribution along river courses. Our specific hypothesis was that *S. emersum* shows a higher potential for endozoochorous seed transport and thus might have a higher capacity for upstream colonization, owing to the higher resistance to gut passage conferred by a hard scleridial endocarp and tough spongy mesocarp, as compared to the soft membranous endocarp and fleshy mesocarp of *S. sagittifolia* seeds. To test this, we performed a gut passage experiment using captive ducks and contrasted its results with those of a field survey where the distribution of both species along two whole river courses was mapped. As a second question we addressed whether different duck species differ in their capacity for endozoochorous seed dispersal. We hypothesized that differences in morphological and physiological properties of the intestinal tract between duck species lead to differences in the digestion and retention of seeds, and hence to differences in potential for endozoochorous seed dispersal (Charalambidou & Santamaria, 2002).

Material & Methods

Study species

S. emersum and *S. sagittifolia* are widely distributed throughout Eurasia and North America (Cook & Nicholls, 1986). In Europe, *S. emersum* and *S. sagittifolia* display similar habitat requirements and are often found together in an association called Sparganieto-Sagittarietum (Cook & Nicholls, 1986; Riis, Sand-Jensen & Vestergaard, 2000; Burkart, 2001). They are typically found together in canals and streams characterized by shallow, stagnant to slow flowing, nutrient-rich freshwaters with sandy or muddy bottoms. However, in river systems, *S. emersum* generally displays a wider longitudinal distribution compared to *S. sagittifolia*, despite the presence of suitable slow-flowing habitats. The drupe-like fruit of *S. emersum* consists of a seed enclosed in a hard scleridial endocarp and a tough spongy mesocarp, with a plugged micropyle (Cook, 1962; Cook, 1996). The fruit of *S. sagittifolia* consists of a nutlet-like seed surrounded by a soft membranous endocarp and a fleshy, semi-transparent, laterally compressed disc-like mesocarp.

Sparganium and *Sagittaria* fruits (hereafter called seeds) are important food for many waterfowl species. To study the potential for endozoochorous seed dispersal of both plant species by waterfowl, we selected mallard *Anas platyrhynchos* (Linnaeus, 1758) and teal *Anas crecca* (Linnaeus, 1758). During fall and winter, the period of seed release and seed dispersal of *S. emersum* and *S. sagittifolia*, they are among the most wide spread waterfowl species in The Netherlands (Voslamber, van Winden & van Roomen, 1998; Devos, 2001), using streams and rivers as their winter habitat (Van Noorden, 1992). During this period, their diet consists mainly of seeds and plant fragments of aquatic plants, grasses and sedges, including seeds of *Sparganium* spp. and *Sagittaria* spp. (McAtee, 1918; Metcalf, 1931; Martin & Uhler, 1939; Anderson, 1959; Nummi, 1993; Mueller & Van der Valk, 2002; Green *et al.*, 2002). Moreover, they display local migratory movements within a home-range (*e.g.* diurnal feeding migrations; Guillemain, Houte & Fritz, 2000, 2002; Mack, Clark & Howerter, 2003), potentially

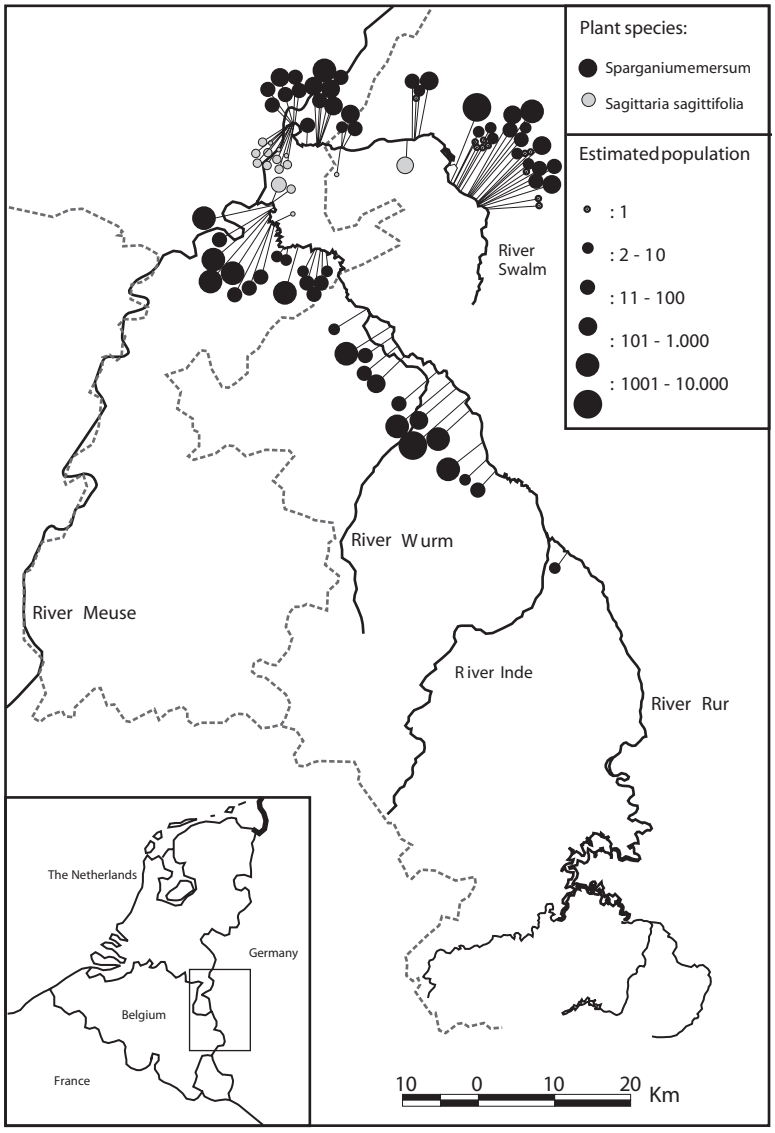

Fig 1 The location of the study area in North-Western Europe and the distribution of *S. emersum* (black dots) and *S. sagittifolia* (gray dots) in the Swalm and Rur rivers and their tributaries. The sizes of the dots are proportional to the number of individuals (upper right-hand panel). The dashed lines represent the country borders.

allowing for seed dispersal away from the plant populations, along the longitudinal axis of the river.

Site description

The River Rur (catchment surface area of 2,340 km^2) originates in the Ardennes Mountains near the Belgian border (at 650 m above sea level), floats through Germany (143.5 km) and The Netherlands (21.5 km), where it discharges in the River Meuse (at 16.8 m above sea level). The channel width varies between 20 to 40 m. The seasonal hydrology is highly dynamic, with discharge ranging from 9.5 to 123 m^3/s, water velocity from 0.2 to 1.3 m/s, and water depth from 2 to 3 m. The River Rur has two smaller side rivers, the Inde and the Wurm, which also originate in the Ardennes near the Belgian border (Fig. 1). The River Swalm (catchment surface area of 277 km^2) originates near the

city of Wegberg (Germany) (at 85 m above sea level), flows through Germany (31 km) and The Netherlands (12.2 km), where it discharges into the River Meuse (at 14 m above sea level). The channel width varies between 3 to 10 m, discharge ranges from 0.5 to 15 m^3/s, water velocity from 0.1 to 1.0 m/s, and channel depth from 0.3 to 1.0 m.

Field survey

During 7-28 July 2003, the entire longitudinal courses of the Swalm and Rur Rivers (and their tributary streams) in Germany and The Netherlands (Fig. 1), were surveyed by boat (in wider and deeper stretches) or by wading through or walking along the river (in smaller and shallower stretches). The water was clear and visibility high (the bottom of the rivers was usually visible, except in certain stretches of the Rur river, deeper than 2,5 meters), allowing the monitoring of submerged plants by visual census. Geographic locations and approximate number of individuals (as estimated on a log scale: 1=1 individual, 2=2-10 individuals, 3=11-100, etc.) of *S. emersum* and *S. sagittifolia* populations were recorded on detailed geographic maps (1:10.000), which contained enough landmarks to pinpoint the exact locations of each population. During the field survey, ducks observed were also recorded.

Experimental design

Ripe seeds of *S. sagittifolia* and *S. emersum* were collected during autumn 2001 from natural populations in the Netherlands. Earlier tests showed that seeds of both species needed to be stratified (*i.e.* subjected to cold temperature while being imbibed in water) for an extended period to break seed dormancy (see also Muenscher, 1936). Therefore, the seeds were stored in glass jars filled with tapwater, in a dark cold room at 5±1ºC for 6 months, to mimic natural stratification conditions of Central-Northern European winters.

We used 10 mallard and 10 teal in our feeding experiments. Mallard had been captured in the wild one year before, while teal were born in captivity and obtained from Kooij & Sons Waterfowl Breeding Farm, The Netherlands. Prior to the experiments, all individuals were housed in outdoor waterfowl facilities (Centre for Terrestrial Ecology, NIOO-KNAW at Heteren, The Netherlands) and kept on a stable diet of commercial pellets (Anseres 3 ® Kasper Faunafood, Waalwijk) and mixed grains (Havens Voeders, Maashees). In the experiment, an even number of males and females of each duck species was used to detect potential differences in digestion and retention between genders. There were significant differences in animal weight between the duck species and genders, with mallard (mean ± SE: males 1.164±0.04 kg, n=5; females 1.003±0.03 kg, n=5) weighing approximately four times as much as teal (males 0.268±0.01 kg, n=5; females 0.258±0.01 kg, n=5).

At the beginning of the feeding experiment, each duck was force-fed 200 seeds (100 *S. emersum* seeds and 100 *S. sagittifolia* seeds). To ensure randomisation, groups of 10 seeds were haphazardly taken from the complete batch of seeds and randomly assigned to the individual ducks. To facilitate

force-feeding, seeds were placed in soft pellets made from Anseres 3 ® food pellets soaked in water. The pellets were placed one by one on the posterior part of the tongue and pushed down the pharynx, subsequently allowing the ducks to swallow the pellet. Immediately after the feeding, each individual duck was transferred to a separate wooden cage (0.6x0.5x0.5 m) where it was kept for the duration of the experiment. Water and food were provided ad libitum throughout the experiments. Produced droppings fell through a maze bottom (square mesh size 13 mm) into plastic containers, which were emptied at four-hour intervals for a period of 60 hours. Collected droppings were immediately rinsed with tap water and sieved using a 500µm square mesh size sieve (diameter of 19cm). Retrieved seeds were transferred to plastic containers (100 ml) filled with tap water and stored at 6°C for the remaining duration of the experiment.

The batch of control seeds received a similar pre- and post-experimental treatment as the seeds used in the feeding experiment (*i.e.* placed in soft pellets soaked in water, sieved with tapwater and stored at 5°C for the duration of the feeding experiment) to exclude possible effects of pre- or post-feeding treatment on the seeds. Control seeds were divided further into two treatments: intact seeds (control) versus scarified seeds (scarified). Seed scarification was aimed at simulating the physical effects of gut passage, by removing the seed coat and abrading the endocarp. The treatment was applied to four random batches with 25 seeds each. Intact, non-scarified seeds included 4 random batches with 100 seeds each.

Immediately after the feeding experiment, ingested, scarified and control seeds were set to germinate in a climate chamber with a photoperiod of 16L/8D, a daytime irradiance of 160-180 µmol photons s^{-1} m^{-2} and a day/night temperature cycle of 25/15°C. Seeds were placed in transparent polystyrene microtiterplates (127x82 cm, 96 wells; Omnilabo International BV, Breda, The Netherlands), filled with tap water (one seed per well). Germination, defined as the emergence of the first foliage leaf, was checked daily for a period of 60 days.

Statistical analysis

Differences in total seed retrieval (*i.e.* the proportion of ingested seeds recovered in the droppings) were tested by means of Generalised Linear Modelling using the GLMMIX module of SAS (SAS Institute Inc., 1996). Differences in retention time were tested in a survival analysis by fitting a Cox proportional hazards regression model to the retrieval time (*i.e.* time between ingestion and retrieval, in hours) for each individual seed, using S-Plus 2000 (Mathsoft). In both analyses plant species, duck species, duck gender and their second-order interactions were included as fixed factors, and the effect of different individuals was added to the model as a random (or failtry) effect.

The overall effects of plant species and seed treatment (*i.e.* intact, scarified and duck ingested) on total germination (*i.e.* proportion of seeds that germinated by the end of the germination run) were tested using Generalised Linear models (as above), followed by pairwise, post-hoc tests comparing the different treatments within each plant species (with a $P<0.008$ comparisonwise error rate, after Bonferroni correction). A separate analysis included retention time as a continuous independent variable plus duck species and duck gender as fixed factors, but only for total

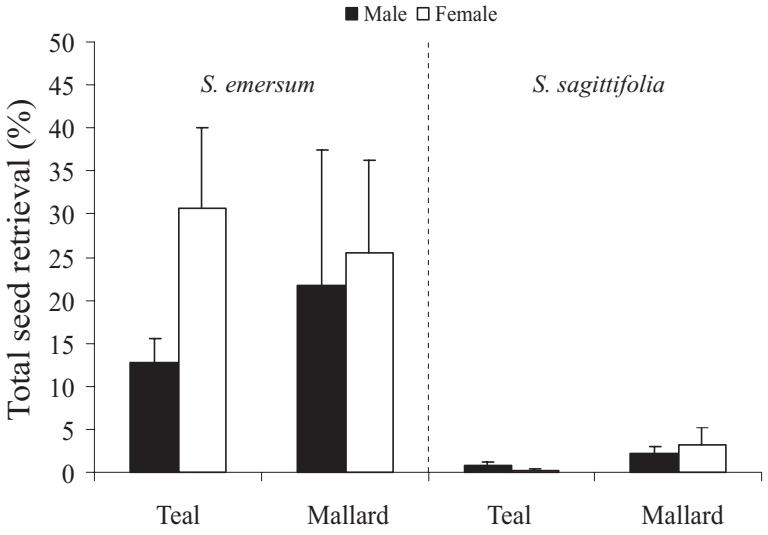

Fig 2 Total seed retrieval (%) over 60 hours, of *S. emersum* and *S. sagittifolia* seeds retrieved from mallard and teal (mean ± SE). Each duck was fed 100 seeds of each plant species. Black bars indicate retrieval from male ducks, white bars from female ducks (n=5 ducks for each gender and species group).

germination of *S. emersum* seeds (due to low retrieval, the analysis could not be performed for *S. sagittaria*). Heterogeneity of slopes was accounted for by selecting the best fit from a family of models that included all possible combinations (as both main factors and interactions) of the categorical factors and the continuous covariate, using the Akaike Information Criterion. Differences in seed germination rates were tested by fitting a Cox proportional hazards regression model to the number of days between setting for germination and seedling emergence, for each individual seed that germinated (non-germinated seeds were excluded from the analysis to separate the effects of germination rate from those on total germination). For each plant species we fitted separate models, which included the same factors for total germination, described above.

Results

Field survey

The upper reach of the Rur (from its origin to the city of Julich, Germany) has a mountainous character, with a high gradient and high water velocity, limiting the presence of aquatic vegetation; thereafter, the gradient is less and plants of the Sparganieto-Sagittarietum association are found in patches of the river with low water velocity (see also Friedrich & Meyer-Holtzl, 2003). The upper reach and several middle parts of the Swalm River are characterized by the presence of dense riparian forests (Carr and Alnus-Betula carr forests) leading to shading of the river bed, and consequently to the absence of aquatic vegetation.

In both rivers, *S. emersum* displayed a wider longitudinal distribution compared to *S. sagittifolia* (Fig. 1). *S. emersum* typically occupied all suitable slow-flowing and shade-free habitat patches

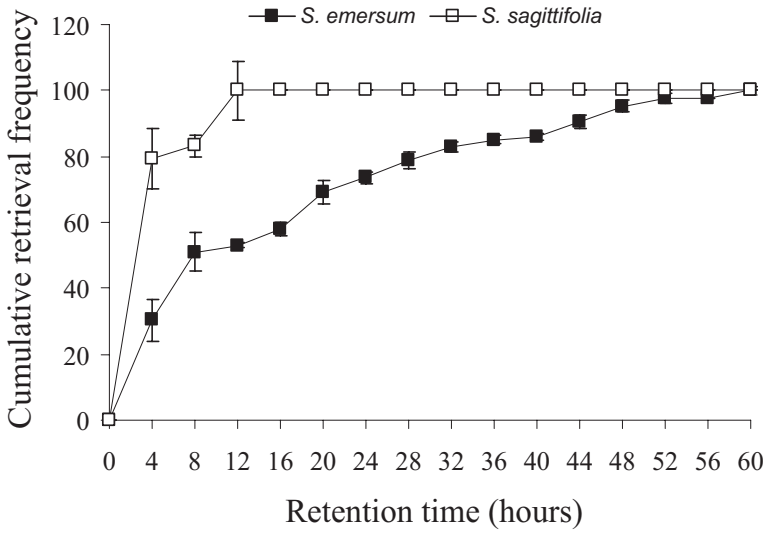

Fig 3 Cumulative retrieval (%) of seeds over time for *S. emersum* and *S. sagittifolia* (mean ± SE; n=20, 10 individuals for both mallard and teal). The experiment was terminated after 60 hours.

along the longitudinal axis in the Rivers Rur and Swalm, while *S. sagittifolia* was restricted to the downstream reaches. Both plant species were absent in the Worm and Inde. During the monitoring of plant populations along both rivers in July, over 230 and 170 mallards were observed along the Rivers Swalm and Rur, respectively. Teal were not observed because they are migratory species, which are absent from The Netherlands during the summer.

Feeding experiments

A significant difference was found in the total seed retrieval between the two plant species ($F_{1,3984}$=19.92, P<0.0001), with retrieval being more than ten-fold higher for *S. emersum* (22.65±20.8%; mean ± SD) than for *S. sagittifolia* (1.60±2.4%). Since the duck faeces contained many seed fragments, especially during the first 8 hours, the rest of the ingested seeds were probably digested in the ducks' digestive tracts. Duck species ($F_{1,3984}$=0.77, P=0.3793) and duck gender ($F_{1,3984}$=1.11, P=0.2931) had no significant effect on the total seed retrieval. The analysis did show significant interaction effects between plant*duck, plant*gender and duck*gender; however, the low number of retrieved *S. sagittifolia* seeds (Fig. 2), precludes a reliable interpretation of these results.

Seed retention time differed significantly between the two plant species (Cox regression: χ^2=43.30, df=1, *P*<0.0001). All *S. sagittifolia* seeds were retrieved within 4-12 hours after ingestion, whereas seeds of *S. emersum* were still retrieved 60 hours after ingestion (Fig. 3), suggesting a potentially much larger range distance of dispersal for *S. emersum*. The pattern of seed retrieval over time followed a leptokurtic curve for both plant species and did not differ significantly between duck species (χ^2=1.14, df=1, *P*=0.290) or gender (χ^2=0.49, df=1, *P*=0.490).

Total germination was significantly affected by plant species, being significantly higher for *S.*

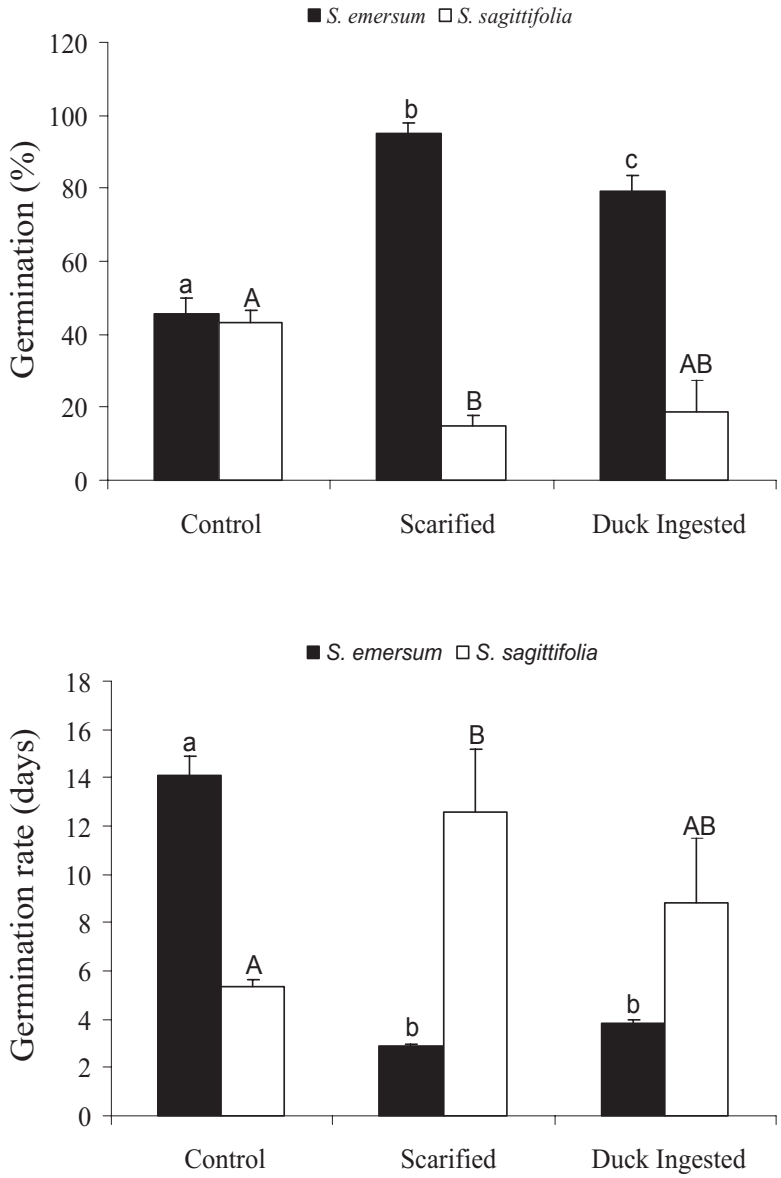

Fig 4 (a) Proportion of germinated seeds (mean ± SE) and (b) day of germination (mean ± SE) of *S. emersum* (black squares) and *S. sagittifolia* (white squares) for the different seed treatments: control (n=400, in 4 batches of 100 seeds), scarified (n=100, in 4 batches of 25 seeds), and duck-ingested (n=453 and 32 respectively, each in 20 batches with variable numbers of seeds). For each species, significant differences between treatments were indicated with letters (a, b, c, for *S. emersum*; and A, B for *S. sagittifolia*). Data points that do not share a common letter are significantly different from each other (see text for P-values).

emersum compared to *S. sagittifolia* ($F_{1,17}$=103.16, P=<0.0001). For *S. emersum*, control seeds showed lower total germination than duck-ingested and scarified seeds ($F_{1,925}$ =8.41 and 15.4, P=0.0038 and 0.0001 respectively), which did not differ significantly from each other ($F_{1,925}$=4.94, P=0.0265; with a comparison-wise error rate of 0.008 after Bonferroni correction, for all three comparisons). For *S. sagittifolia*, control seeds showed higher total germination than scarified seeds ($F_{1,152}$ =24.01, P=<0.0001), whereas germination of duck-ingested seeds was intermediate between that of control and scarified seeds, and did not differ significantly from either (P>0.008, Fig. 4a).

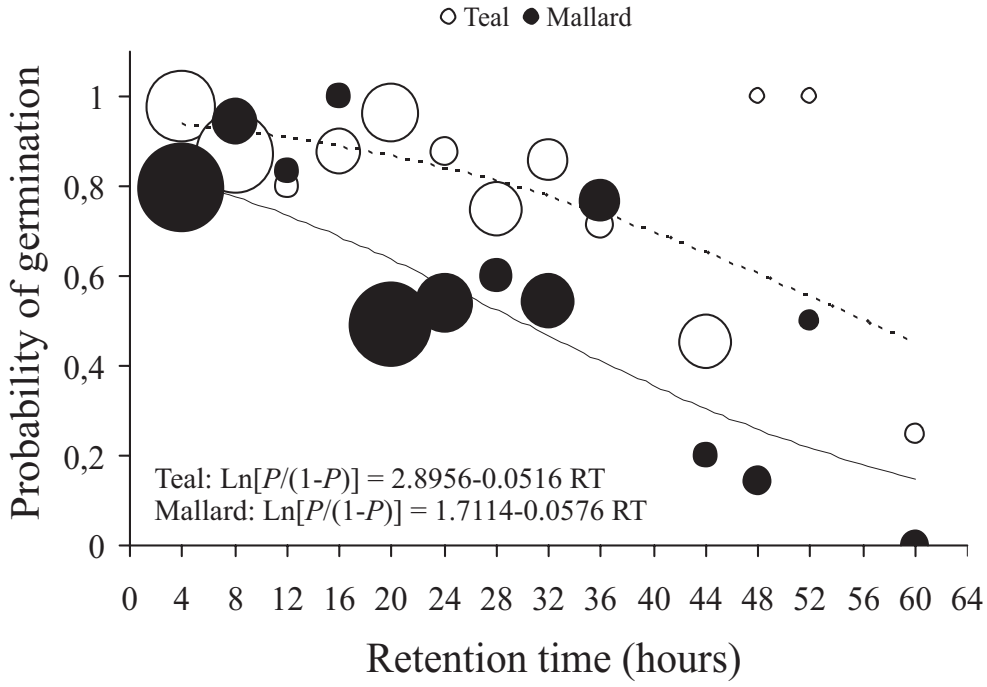

Fig 5 Effect of retention time in the digestive tract of mallard (black dots) and teal (white dots) on the probability of germination of retrieved *S. emersum* seeds. Displayed equations were obtained from logistic regression after backward removal of non-significant factors. The sizes of the dots are proportional to the number of seeds, ranging from 7-98.

Retention time significantly affected the total germination of *S. emersum* seeds, with decreased germination at longer retention times ($F_{1,431}$=3.88, P=0.0495; Fig. 5). Total germination of retrieved seeds differed significantly between duck species, being higher for teal than for mallard ($F_{1,431}$=7.65, P=0.0059).

For *S. emersum*, control seeds displayed slower germination rate (*i.e.* number of days to germination) than duck-ingested and scarified seeds (Cox regression: χ^2=117.8 and 86.7; df=1, *P*<0.0001 respectively), which did not differ significantly between each other (χ^2=0.77, df=1, *P*=0.38; Fig. 4b). For *S. sagittifolia*, control seeds had faster germination rates than scarified seeds (χ^2=12.97, df=1, *P*=0.0003), whereas germination rate of duck-ingested seeds was intermediate between that of controls and scarified seeds, and did not differ significantly from any of these (χ^2=0.56 and 7.54, df=1; *P*=0.450 and 0.060 respectively). In a separate analysis on duck-ingested seeds, germination rate of *S. emersum* seeds was neither affected by duck species (Cox regression: χ^2=0.28, df=1, *P*=0.600) nor gender (χ^2=0.50, df=1, *P*=0.480).

Discussion

Differences between plant species

The effect of gut passage on ingested seeds is known to differ among plant species, largely owing to differences in the structure of the seed coat (Proctor, 1968; Traveset, 1998; Traveset & Verdú, 2002). We hypothesized that seeds of *S. emersum* would show higher resistance to gut passage compared to seeds of *S. sagittifolia*, since they are enclosed in a hard scleridial endocarp and a tough spongy mesocarp. Seeds of *S. sagittifolia* have a soft membranous endocarp and fleshy mesocarp and are therefore more likely to be damaged or completely digested during gut passage (Traveset, 1998). The results indeed suggest that *S. emersum* has a higher potential for endozoochorous dispersal compared to *S. sagittifolia*. Firstly, a very small proportion of the ingested *S. sagittifolia* seeds was retrieved (1.60±2.4%), more than fourteen times less than the proportion of *S. emersum* seeds that was retrieved (22.65±20.80%). Secondly, all *S. sagittifolia* seeds were retrieved within the first 12 hours after ingestion, whereas viable *S. emersum* seeds were still retrieved after a retention time of 60 hours when the experiment was terminated, indicating a larger 'window of opportunity' for dispersal events to occur and a potential for much larger dispersal distances for *S. emersum*. Thirdly, retrieved *S. emersum* seeds displayed increased germination and higher germination rates, whereas retrieved *S. sagittifolia* seeds (which had lost the fleshy mecocarp and occasionally part of the membranous endocarp) showed decreased germinability and delayed germination rates, compared to non-ingested control seeds (Table 1).

The reduction in germination and germination rate of retrieved *S. sagittaria* seeds is most likely related to extensive grinding in the ducks' guts, partly bruising the seed embryo and affecting its capacity to germinate. Scarification of *S. sagittifolia* seeds resulted in a similar reduction of seed germination, suggesting that the mechanical treatment, rather then the chemical treatment, in the gut may be responsible for the observed reduction in seed germination following gut passage. On the other hand, retrieved seeds of *S. emersum* displayed a significant increase in germination and germination rate, most likely related to the breaking of the seed-coat dormancy (Baskin & Baskin, 1998), which is necessary before germination can commence (Cook, 1962). Under natural conditions the seed coat of *S. emersum* can be broken after a period of freezing, or by natural decomposition. Passage through the digestive tract of waterfowl may similarly increase germination of seeds with a hard seed coat (Santamaria *et al.*, 2002). In fact, our results show that scarification (*i.e.* manual removal of the seed coat) increases the germination and germination rate significantly, suggesting that the mechanical treatment in the gizzard, that results in removal of seed coats, is responsible for the increased germination of seeds with a hard seed coat. Hence, our results (Table 1) suggest that *S. emersum* has a higher potential for endozoochorous dispersal by ducks and post dispersal establishment, while *S. sagittifolia* is less likely to undergo such a mode of dispersal.

Table 1 Summary of the effect (mean ± SE) of seed ingestion by ducks on the retrieval (RT is the retention time) and germination of seeds of *S. emersum* and *S. sagittifolia*. The effect on germination was compared to intact control seeds.

	Seed structure	Retrieval		Germination	
Plant Species	Endocarp-Mesocarp	Max. RT (hours)	Total retrieval (%)	Germination rate (days)	Total germination (%)
S. emersum	Hard scleridial endocarp and tough spongy mesocarp	60 h*	22.65±20.8	acceleration of 10 days	increase of 33.3 %
S. sagittifolia	Soft membranous endocarp and fleshy mesocarp	< 12 h	1.60±2.4	delay of 3.5 days	decrease of 24.4 %

* Experiment was terminated at 60 h

Differences between duck species

Different duck species are likely to differ in their quality as endozoochorous seed dispersers, due to differences in their ecology, body size, gut morphology and digestive physiology (Charalambidou & Santamaria, 2002; Green, Figuerola & Sanchez, 2002; Figuerola, Green & Santamaria, 2002). However, few studies have looked at differences in retrieval and subsequent germination success of seeds that have been ingested by different duck species.

In the present study, we found no significant difference in the total proportion of retrieved seeds between teal and mallard. The lack of observed inter-specific differences is most likely due to the large intra-specific variation among individuals (with the proportion of retrieved seeds ranging from 4 to 74 %). Significant differences in seed retrieval over retention time between teal and mallard were not observed, despite the fourfold difference in body size between both species. Both species have similar feeding habits, both being opportunistic generalist feeders (Nummi, 1993) that display a seasonal diet shift from predominantly zoobenthivorous in spring and summer to predominantly granivorous in fall and winter. Both ducks can show changes in the size and morphology of their intestinal tract, in order to adapt to seasonal changes in food supply (Whyte & Bolen, 1985; Nummi, 1993). It has been suggested that such closely related *Anas* spp. have similar digestive physiologies (Miller, 1984), and that interspecific differences among these *Anas* spp. (such as differences in body size) may have little effect on retention time and digestion of seeds in their guts (Charalambidou, Santamaria & Langevoord, 2003).

Finally, we did find a significant difference between duck species in the total germination of retrieved *S. emersum* seeds. Total germination (%) of *S. emersum* seeds was slightly (though significantly) higher for seeds retrieved from teal compared to seeds retrieved from mallard. Since germination of *S. emersum* seeds depends largely on the removal of the seed coat (see above), we suggest that the observed differences are due to a less efficient removal of the seed coat by mallard, compared to teal. Interestingly, this difference is: on the one hand, apparently strong enough to lead to a more efficient removal of the tough spongy mesocarp of *S. emersum* seeds in the intestinal tract, and therefore to a higher germination (%) of the retrieved seeds, whereas on the other hand,

it is not strong enough to be more damaging to the hard scleridial endocarp of *S. emersum* seeds, and hence does not lead to a difference in the total retrieval (%) of *S. emersum* seeds between duck species.

A mechanism for plant population persistence in rivers

The theory of what has become known as the ´drift paradox´ was first formulated to describe the chronobiology of mobile stream organisms, such as invertebrates, amphibians and fish (Müller, 1974; Hersey *et al.*, 1993; Williams & Williams, 1993; Anholt, 1995). The theory states that population persistence in rivers depends on active upstream movement of individuals to compensate for the loss of individuals due to downstream drift (Speirs & Gurney, 2001). However, many aquatic species, including aquatic macrophytes, lack a means of active upstream dispersal and for them the paradox of population persistence remained unresolved.

In this study, we investigated the potential for endozoochorous seed dispersal by waterfowl species. Although we did not conduct specific observations on the feeding behaviour of mallard and teal, we did observe that in October 2003 the ducks were often associated with plant populations of both species. Since during autumn and winter they are predominantly granivorous feeders (McAtee, 1918; Metcalf, 1931; Martin & Uhler, 1939), ingestion of *S. emersum* and *S. sagittifolia* seeds by mallard and teal was likely to occur. Moreover, a preliminary study on the genetic population structure of *S. emersum* in the River Swalm, using microsatellites, provided some evidence for the occurrence of colonisation events to upstream areas (de Jong, 2004). We propose that plant population persistence in rivers is a dynamic process over a large time-scale, driven by two opposing forces: a steady downstream movement of populations due to unidirectional currents (Speirs & Gurney, 2001), counteracted by (possibly infrequent) upstream colonisation events due to animal-assisted dispersal. Particularly in rivers, animal-mediated seed dispersal may constitute an important component of dispersal of plants, through directional dispersal of seeds to empty niches in upstream areas. It has been suggested, that even infrequent upstream dispersal events would allow population persistence in river ecosystems, because depopulated upstream areas provide empty niches with little competition ensuring increased individual fitness to new colonists (Schupp, 1993; Anholt, 1995; Higgins, Nathan & Cain, 2003; Levine, 2003).

The present study is the first to implicate the potential importance of animal-mediated dispersal for plant population persistence in rivers. If so, differences between plant species in their ability to colonise upstream areas, may have consequences for their distribution in a river: species with a high potential of animal-mediated upstream dispersal and post-dispersal establishment (*i.e. S. emersum*) may display a wider longitudinal distribution, compared to ´badly dispersed species´ (*i.e. S. sagittifolia*), assuming that these areas provide suitable habitats for the plant species and animal vectors. The results from the field survey show that in the rivers Ruhr and Swalm (Fig. 1), *S. emersum* indeed displays a higher upstream distribution compared to *S. sagittifolia* (Fig. 1). However, this single field survey provides only preliminary and circumstantial evidence for bird-mediated dispersal, and the mechanisms that determine population persistence in rivers require further research.

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Chapter 5

Isolation and characterization of microsatellites in *Sparganium emersum* and cross-species amplification in the related species *S. erectum*

BJA Pollux & NJ Ouborg

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Summary

We developed seven novel polymorphic microsatellite loci for the aquatic macrophyte Sparganium emersum (Sparganiaceae). These were characterized on 62 individuals collected from nine different populations. In this set of individuals, 7 to 20 alleles per locus were detected and observed heterozygosity ranged between 0.16 and 0.95. Cross-species amplification was tested in the related species Sparganium erectum, and was successful for five of the seven microsatellite loci.

Rivers offer special environments for plant dispersal, because of their uni-directional water flow and linear arrangement of suitable habitats. Gaining insight in the functioning of riparian plant populations requires studying the rate and direction of dispersal, as well as the contribution of seed dispersal compared to the dispersal of clonal structures (Ouborg *et al.* 1999).

Unbranced bur-reed, *Sparganium emersum* Rehmann 1871 (Sparganiaceae), is a monoecious aquatic macrophyte that is commonly found in rivers and streams throughout Eurasia and North America. *Sparganium emersum* is a wind-pollinated species, whose seeds are mainly dispersed by water currents (Boedeltje *et al.* 2004) and, less frequently, by waterfowl species (Pollux *et al.* 2005). The plant reproduces vegetatively via the production of stolons from which new ramets emerge. Occasionally, plant parts may break off, be dispersed by water currents and become successfully established in new locations (Barrat-Segretain & Bornette 2000). These characteristics, common to many other riparian species, make *S. emersum* a useful species for studying the functioning of riparian plant populations in rivers. Here we present the characterization of seven polymorphic microsatellite loci, suitable for the study of gene flow and the genetic structure of *S. emersum* populations in rivers.

Di-, tri- and tetranucleotide repeat enriched libraries of *S. emersum* genomic DNA were constructed by a selective hybridisation procedure (Karagyozov *et al.* 1993), using the method described by Arens *et al.* (2000, 2004) with minor modifications. Genomic DNA of five individuals was isolated using the DNeasy® Plant Mini Kit (Qiagen). Genomic DNA was restricted with *Mbo*I (MBI Fermentas) and size-fractionated by agarose gel electrophoresis. DNA fragments from 300 to 1250 bp in size were extracted from the agarose gels using the QiaEx II Gel Extraction Kit (Qiagen), and enriched by hybridisation to either single di- or trinucleotide repeats $(GA)_{12}$, $(GT)_{12}$, $(AGT)_9$, $(TGA)_9$, $(TGT)_{10}$, $(TCT)_{10}$, a pool of trinucleotides [$(GAG)_8$, $(GTG)_8$, $(CGT)_8$, $(GCC)_7$, $(GCT)_8$, $(TAA)_{12}$)], or a pool of tetranucleotides [$(TGTT)_8$, $(CTAT)_8$, $(GATA)_8$, $(GACA)_8$, $(GGAT)_7$, $(TCTT)_8$]. Enriched fragments were ligated into the pGEM®-T Easy Vector System I (Promega) and transformed into *Escherichia coli* DH5α competent cells. Colonies were transferred onto Hybond N+ membranes (Amersham-Pharmacia). Phage filters were probed with a set of ^{32}P-labeled synthetic repeat polynucleotides, and positive clones were identified by autoradiography. Sixty-four positive clones (1.3% of the total number) were obtained, which were all sequenced on a model 4000 L DNA Sequencer (Li-cor) using the SequiTherm EXEL™ II DNA Sequencing Kit-LC (Epicentre Technologies) and IR-800 dye-labelled M13 primers (Biolegio).

Thirteen sequences were selected for microsatellite primer development, eight containing a $(GA)_n$ repeat and the remaining five containing either a $(CA)_n$, $(TCC)_n$, $(GTT)_n$, $(AGT)_n$ or a $(CTAT)_n(CT)_n$ repeat. Primer pairs were designed using PrimerSelect™ (DNAstar) and synthesized by Biolegio (Malden, The Netherlands). The markers were tested and optimised using different $MgCl_2$ concentrations and annealing temperatures (50-70 °C on a T-Gradient, Biometra®). The amplification reactions were performed in a twenty-five µl volume containing 20 ng template DNA, 1.25 x NH_4 Bioline Buffer, 0.25 mM dNTPs, $MgCl_2$ as in Table 1, 0.05 µM of each primer (forward primers were IR-800 dye-labeled), and 1 Unit BioTaq Red™ DNA Polymerase (Bioline). Amplification was carried out in a T3 Thermocycler (Biometra®) with the following thermal profile: 1 initial cycle of 5 min at 94 °C, followed by 40 cycles of 45 s at 94 °C, 45 s at annealing

Table 1 Characterization of seven microsatellite loci in *Sparganium emersum*, tested on 62 individuals from 9 populations in the Netherlands. Primer sequences (F, forward; R, reverse), $MgCl_2$ concentration, annealing temperature (T_a), repeat motif, allele size range, number of alleles per locus (A), observed heterozygosity (H_O) and expected heterozygosity (H_E).

Locus	GenBank Accession no.	Primer sequence (5'-3')	$MgCl_2$ (mM)	T_a (°C)	Repeat motif	Size range (bp)	A	H_O	H_E	Cross-species amplification in *S. erectum*
SEM01	DQ304634	F: GTCGGAGCCCTCTGCCTTCA	2.5	53	$(GA)_n$	153-171	9	0.754	0.659	+*
		R: TTCATGTAAATTGGTTGTCTTCA								
SEM05	DQ304636	F: TACACTTTCCTATCCCCATTCA	1.25	52	$(GA)_n$	303-349	17	0.952	0.791	+ +†
		R: CCAAAGCCAAAACAAGATACC								
SEM08	DQ304638	F: GTGGTGGCGATGGCAATAAT	2.5	56.4	$(GA)_n$	151-183	14	0.873	0.861	+ +
		R: CAAGGTAGTGGCGACAAG								
SEM12	DQ304640	F: CAGGCCGGTTGGACAGGTAGTT	1.25	60	$(CTAT)_n(CT)_n$	215-265	9	0.495	0.517	-
		R: GGGAAGAGCAGCCAAGACGAAGTA								
SEM14	DQ304642	F: TTCATGTAAATTGGTTGTCTTC	2.5	60	$(GA)_n$	225-239	7	0.823	0.674	+†
		R: TCCCCTACTTCCCCTAATCGTTGTC								
SEM15	DQ304643	F: GGCGTGGACGTGGGTGGTGT	2.5	61.6	$(GA)_n$	234-274	13	0.794	0.767	+ +
		R: CATTGGGCTAGTAGGCTTGTTTAT								
SEM17	DQ304644	F: CACATACGCCACTGCTTTTT	2.5	60	$(GA)_n$	201-285	20	0.159	0.845	-
		R: TTTTTCCCGCGCTCTCAAC								

Amplification test on three individuals of *S. erectum*: + +, good amplification; +, weak amplification; -, no amplification.
* Indicates polymorphic bands between individuals.
† Indicates individuals with a homozygote band pattern.

temperature as in Table 1, and 1 min at 72 °C, followed by a final extension step of 10 min at 72 °C. Fragments were analysed on a model 4200 IR^2 DNA Analyser (Li-cor) using the $Saga^{GT}$ Automated Microsatellite Analysis Software Version 2.1 (Li-cor).

Out of 13 tested markers, 7 primer pairs gave reproducible well-scorable PCR products of the expected size (Table 1). The seven primer pairs were tested on 62 plants collected from 9 populations in the Swalm and Rur rivers (the Netherlands). Heterozygosity values for each locus were calculated using FSTAT Version 2.9.3 (Goudet 1995). Observed heterozygosities were generally high and deviations from expected heterozygosities small, with the exception of SEM17 (Table 1). The relatively large deviation in SEM17, indicating an excess of homozygotes, could be due to inbreeding, the Wahlunds' effect or the presence of null alleles. However, since the deviations in the other loci are generally small, inbreeding and the Wahlunds' effect are unlikely, suggesting that the locus SEM17 may potentially contain null alleles. The Fisher's Exact test for Hardy-Weinberg equilibrium using Genepop Version 3.4 (Raymond & Rousset 1995), showed that three out of 8 loci were not in HWE (SEM07, SEM08 and SEM17; $P<0.05$). A significant genotypic association was found for two out of the 28 pairs of loci: SEM1-SEM12 ($P=0.001$) and SEM01-SEM08 ($P=0.003$). Results show that the microsatellites will be a useful tool for studying dispersal and the genetic structure of *S. emersum* populations. Finally, the primers were tested for cross-species amplification on the related species *S. erectum*, using 3 individuals from 2 different populations. Succesful amplification was observed for five of the seven loci (Table 1).

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Chapter 6

Reproductive strategy, clonal structure and genetic diversity in populations of the aquatic macrophyte *Sparganium emersum* in river systems

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Summary

Many aquatic and riparian plant species are characterized by the ability to reproduce both sexually and asexually. Yet, little is known about how spatial variation in sexual and asexual reproduction affects the genotypic diversity within populations of aquatic and riparian plants. We used six polymorphic microsatellites to examine the genetic diversity within and differentiation among 17 populations (606 individuals) of Sparganium emersum, in two Dutch-German rivers. Our study revealed a striking difference between rivers in the mode of reproduction (sexual vs asexual) within S. emersum populations. The mode of reproduction was strongly related to locally reigning hydrodynamic conditions. Sexually reproducing populations exhibited a greater number of multi-locus genotypes compared to asexual populations. The regional population structure suggested higher levels of gene flow among sexually reproducing populations compared to clonal populations. Gene flow was mainly mediated via hydrochoric dispersal of generative propagules (seeds), impeding genetic differentiation among populations even over river distances up to 50 kilometres. Although evidence for hydrochoric dispersal of vegetative propagules (clonal plant fragments) was found, this mechanism appeared to be relatively less important. Bayesian-based assignment procedures revealed a number of immigrants, originating from outside our study area, suggesting inter-catchment plant dispersal, possibly the result of waterfowl-mediated seed dispersal. This study demonstrates how variation in local environmental conditions in river systems, resulting in shifting balances of sexual vs asexual reproduction within populations, will affect the genotypic diversity within populations. This study furthermore cautions against generalizations about dispersal of riparian plant species in river systems.

Introduction

Rivers offer special environments to aquatic and riparian plants, due to the one-dimensional linear arrangement of suitable habitats, the continuous subjection to the hydraulic forces of water currents and the uni-directional nature of the water flow. Knowledge about the processes that determine the genetic structure of populations (*e.g.* life form, reproductive biology, clonal propagation, dispersal mechanisms) is essential for understanding the scale over which dispersal, genetic drift and selection operate (Slatkin 1985; Heywood 1991; Ouborg *et al.* 1999).

Most aquatic and riparian plant species are characterized by the ability to reproduce sexually via seeds, and asexually via stolons, runners, tubers, etc. (Barrett *et al.* 1993). Some studies suggest that the age of plant populations affects the mode of reproduction, although opposing views exist on the underlying mechanisms that might determine the mode of reproduction (Piquot *et al.* 1998; Sun *et al.* 2001). Other studies have shown that the relative proportions of sexual *vs* asexual reproduction varies widely within a plant species, due to variations in environmental parameters (Honnay & Bossuyt 2005). Within the geographical range of a species, for example, plants may increasingly suffer from physiological stress near the boundaries of their geographical range, leading to reduced sexual reproduction (decreased flower, fruit and seed production) or seedling recruitment (Cox & Moore 1980; Dorken & Eckert 2001; Eckert 2002; Lui *et al.* 2005). In temperate deciduous forests, moreover, the relative investment in sexual *vs* clonal reproduction has been shown to vary in response to spatial heterogeneity of light conditions and soil moisture content: Kudoh *et al.* (1999) found that sexual reproduction of *Uvularia perfoliata* was restricted to high-light conditions (in gap sites), whereas under low-light conditions (in closed-canopy sites) plants reproduced clonally; and Jacquemyn *et al.* (2006) showed that sexual reproduction of *Paris quadrifolia* was primarily found in moist and relatively productive sites, while under stressful conditions (*i.e.* in dry and relatively unproductive sites) sexual reproduction and seedling recruitment was suppressed. In aquatic systems, spatial variation in water depth and current velocity have also been known to affect the mode of reproduction within populations of several different plant species, by limiting the plants' ability to produce emergent flower-bearing stems in deep habitats or fast-running streams (Haslam 1978; Boeger & Poulson 2003; van Wijk 1988).

The mode of reproduction (sexual *vs* asexual) is likely to have important effects on the spatial distribution of genetic variation within and among plant populations in rivers (Ellstrand & Roose 1987; Widén *et al.* 1994; Honnay & Bossuyt 2005). Firstly, sexual reproduction is likely to enhance the level of gene flow among populations via seed dispersal. The level of connectivity among riverine plant populations will, to a large extent, determine their genetic structure (Tero *et al.* 2003). In plant species with hydrochory as their main dispersal strategy, unidirectional gene flow may be expected to lead to erosion of genetic diversity in upstream river stretches and accumulation of genetic diversity in downstream stretches (Barrett *et al.* 1993). Such associations have, however, rarely been found (Gornall *et al.* 1998; Lundqvist & Andersson 2001; Liu *et al.* 2006). Secondly, the occurrence of modular clonal units (ramets) originating from the same sexually produced offspring (genets) will directly affect the genotypic diversity within populations (Ellstrand & Roose 1987; Widén *et al.* 1994; Honnay & Bossuyt 2005). Thus, insight into how spatial variation in

sexual and asexual reproduction varies across environmental parameters will help understanding the genetic structure of (facultatively clonal) plant populations in river systems.

In this study, we employed microsatellite analysis to examine the genotypic diversity within and genetic differentiation among 17 populations of *S. emersum* in two different rivers, the Swalm and Rur rivers (Germany - the Netherlands). These two rivers differ widely in their hydrodynamic regime. Several studies have shown that aquatic and riparian plants respond to increased water velocities through plastic morphological changes in order to reduce mechanical damage (Chambers *et al.* 1991; Schutten & Davy 2000), affecting their ability for sexual reproduction (Haslam 1978; Boeger & Poulson 2003). We hypothesized that spatial variation in current velocity within and between river systems would affect the mode of reproduction within populations, in turn affecting the intrapopulation genotypic diversity. The objectives of this study were, therefore, to determine: (i) how hydrodynamic conditions experienced by the plant populations affect their morphology, and consequently their ability for sexual *vs* asexual reproduction, (ii) the extent and patterns of microsatellite variability within and among *S. emersum* populations, and (iii) whether the genetic and genotypic diversity within populations reflects a local balance between sexual and asexual reproduction.

Material and methods

Study species

Unbranched burreed, *Sparganium emersum* Rehmann 1871 (= *S. simplex* Hudson 1778) (Sparganiaceae), is an aquatic vascular macrophyte, that is widely distributed throughout Eurasia and North America (Cook & Nicholls 1986). It typically grows in a narrow band at the margins of rivers, streams and canals that are characterized by shallow, slow flowing, nutrient-rich waters. *S. emersum* is a monoecious and protandrous species (Sargent & Otto 2004). *S. emersum* flowers from June to August, and its flowers are mainly wind-pollinated (Sargent & Otto 2004). The seeds are released in autumn and are mainly dispersed by water currents and waterfowl species (Boedeltje *et al.* 2004; Pollux *et al.* 2005). Vegetative plant fragments are also dispersed by water currents, remaining viable and capable of establishment even after floating for up to 10 weeks (Barrat-Segretain & Amoros, 1996; Barrat-Segretain *et al.* 1998, 1999). *S. emersum* is also capable of asexual (clonal) reproduction through the production of stolons, from which new ramets emerge (Cook & Nicholls 1986).

Study sites

The River Rur (catchment surface area of 2,340 km^2; Figure 1) originates in the Ardennes Mountains near the Belgian border (at 650 m above sea level), floats through Germany (143.5 km) and The Netherlands (21.5 km), where it discharges in the River Meuse (at 16.8 m above sea level). The channel width varies between 20 to 40 m. The seasonal hydrology is highly dynamic, with discharge ranging from 9.5 to 123 $m^3 \cdot s^{-1}$. The profile of the channel bed is characterized by gradually sloping

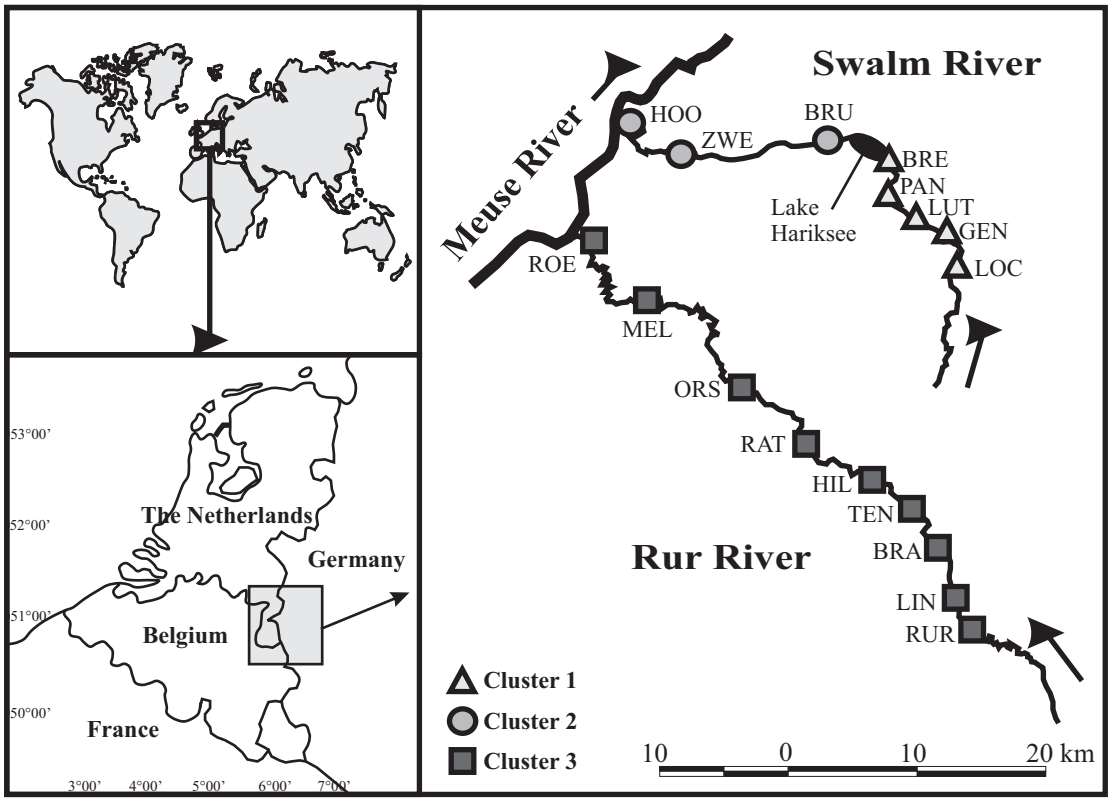

Fig 1 Map showing the 17 sampling locations of the unbranched burreed (*Sparganium emersum*) in the Swalm and Rur rivers (the Netherlands-Germany). The three different clusters, inferred from the Bayesian clustering analysis, have been indicated by different symbols.

river banks running down to a depth of 2 m. The River Swalm (catchment surface area of 277 km^2; Figure 1) originates near the city of Wegberg (Germany) (at 85 m above sea level), flows through Germany (31 km) and The Netherlands (12.2 km), where it discharges into the River Meuse (at 14 m above sea level). The channel width varies between 3 to 10 m and discharge ranges from 0.5 to 15 m^3 ·s^{-1}. The profile of the channel bed is characterized by steep slopes and a uniform channel depth of approximatly 0.5 m. In the middle of its course lies Lake Hariksee, a large shallow lake formed after peat excavations in the 19th century.

Field survey

During 12-23 September 2005, plant density and proportion of flowering plants (%) were determined for each of the 17 study populations by counting the number of plants, and the number of flowering plants, within five randomly selected 0.4 x 0.4 m square areas in each population. Plant biomass was assessed by measuring the dry weight, on a micro-balance (Sartorius LP620P), of 10 randomly collected plants after oven-drying for 24 hours at 55°C. The number of seeds and seed heads per plant were inferred from up to 10 flowering plants randomly collected from each population. In addition, water velocities among plants were determined by measuring water

velocity at five randomly selected locations within each plant population, at 5 cm below the water surface, using a SENSA-RC2 Water Velocity Meter (Aqua Data Services Ltd., Aquatec House, Lyneham, Chippenham, Wiltshire, UK). Differences in plant density and plant biomass between rivers were tested for significance by means of a One-Way ANOVA. Prior to the analyses, all data were log10-transformed to assure homoscedasticity and normality of residuals. All analyses were performed with STATISTICA 6.0 (StatSoft Inc, Tulsa, Oklahoma, USA).

Sample collection, DNA extraction, PCR amplification and microsatellite analysis

In July 2003, a total of 606 *S. emersum* plants were collected from 8 discrete locations in the Swalm river and 9 discrete locations in the Rur river (Figure 1, Table 1). In each site, plants were collected at 1 to 2 m intervals along a linear transect running parallel to the shore. Plant samples were immediately transferred to a 1.5 ml eppendorf tube and stored at -80°C until the DNA extraction. Genomic DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen). Each individual was screened using 6 microsatellite primer pairs (SEM01, SEM05, SEM08, SEM12, SEM14 and SEM15; Pollux & Ouborg 2006). Fragments were analysed on a model 4200 IR^2 DNA Analyser (Li-cor) using the Saga^{GT} Automated Microsatellite Analysis Software Version 2.1 (Li-cor).

Genetic and genotypic diversity

We used a number of standard measures to describe the clonal structure of each population. The proportion of distinguishable genets was calculated as: $P=G/N_r$, where G is the number of distinguishable genotypes and N_r the total number of sampled ramets (Ellstrand & Roose 1987). Second, for each population we determined the number of local (*i.e.* unique) genotypes (G_L) (Ellstrand & Roose 1987). Third, we calculated Simpson's diversity index (D; Simpson 1949) corrected for finite sample sizes as: $D=1-\Sigma\{[n_i(n_i-1)]/[N_r(N_r-1)]\}$, where n_i is the number of individuals with the same genotype and N_r the number of ramets sampled (Widén *et al.* 1994). A Mann-Whitney U test was used to assess whether genotypic variation within populations (G, G_L, P, and D) differed between rivers. The number of unique genotypes possible was calculated as: $N_g=\Pi[a_i(a_i+1)]/2$, where a_i is the number of alleles detected at the i^{th} locus (Widén *et al.* 1994). In addition, we calculated the probability that two individual ramets with the same multi-locus genotype originated from the same genet as: $P_{gen}=(\Pi\, p_i q_i)2^h$, where p_i and q_i is the frequency of the two alleles at the i^{th} locus and h is the number of heterozygous loci represented in the genotype (Parks & Werth 1993; but see Gregorius 2005). If $P_{gen} < 0.001$ for a given genotype, then ramets carrying this genotype were assigned to the same genet. Recurring genotypes within populations were excluded from all further analyses.

The number of alleles (A) and expected and observed heterozygosity (H_E and H_O) were obtained using the POPGENE version 1.31 computer program (Yeh *et al.* 1997). GENEPOP version 3.4 (Raymond & Rousset 1995) was used to calculate the inbreeding coefficient (F_{IS}) for each locus in each population, and to test for linkage disequilibrium for all pairs of loci. Conformance

Table 1 Population characteristics (mean ± SD) and genotypic diversity statistics for the 17 *Sparganium emersum* populations in the Swalm and Rur rivers (N_r = number of ramets sampled in each population, G = number of unique genotypes idendified, G_L = the number of local, *i.e.* unique, genotypes, P = the proportion of distinguishable genotypes and D = Simpson's diversity index).

River	Population		Population characteristics						Genotypic diversity				
			Water velocity ($m\ s^{-1}$)	Plant density (m^{-1})	Plant biomass (g)	Proportion flowering plants (%)	Number of seed heads	Number of seeds	N_r	G	G_L	P	D
Swalm	1	LOC	0.384 (0.09)	243.8 (91)	0.54 (0.4)	0	0	0	30	1	0	0.033	0
	2	GEN	0.480 (0.03)	281.3 (72)	0.31 (0.3)	0	0	0	33	2	1	0.061	0.1174
	3	LUT	0.547 (0.09)	327.1 (103)	0.23 (0.1)	0	0	0	33	1	0	0.030	0
	4	PAN	0.447 (0.06)	343.8 (27)	0.38 (0.2)	0	0	0	35	1	0	0.029	0
	5	BRE	0.373 (0.08)	387.5 (147)	0.41 (0.2)	0	0	0	35	11	11	0.314	0.5731
	6	BRU	0.333 (0.10)	183.3 (22)	0.45 (0.2)	0	0	0	33	13	13	0.394	0.8845
	7	ZWE	0.507 (0.13)	214.6 (19)	0.51 (0.2)	0	0	0	35	1	0	0.029	0
	8	HOO	0.260 (0.12)	243.8 (50)	0.44 (0.2)	0	0	0	35	1	0	0.029	0
Rur	1	RUR	0.060 (0.01)	95.2 (21)	1.97 (1.3)	0.44 (0.2)	2.67 (0.8)	188.43 (75.4)	20	1	1	0.050	0
	2	LIN	0.058 (0.02)	97.9 (53)	1.77 (0.5)	0.39 (0.1)	3.00 (0.7)	139.10 (111.2)	40	37	35	0.925	0.995
	3	BRA	0.042 (0.01)	133.3 (38)	1.73 (0.5)	0.23 (0.1)	3.22 (0.7)	233.67 (60.5)	40	36	30	0.900	0.991
	4	TEN	0.029 (0.00)	120.8 (51)	2.24 (1.0)	0.66 (0.0)	2.80 (0.6)	170.00 (64.7)	40	38	30	0.950	0.997
	5	HIL	0.052 (0.01)	100.7 (34)	2.60 (0.7)	0.30 (0.2)	4.00 (1.0)	412.12 (137.0)	39	21	18	0.538	0.896
	6	RAT	0.113 (0.03)	120.8 (42)	0.69 (0.2)	0	0	0	40	32	27	0.800	0.988
	7	ORS	0.115 (0.04)	72.9 (22)	1.60 (0.3)	0.21 (0.1)	2.83 (0.7)	114.67 (43.2)	39	30	23	0.769	0.974
	8	MEL	0.047 (0.01)	104.2 (38)	2.84 (0.9)	0.32 (0.1)	3.29 (0.5)	197.14 (79.5)	39	32	29	0.821	0.989
	9	ROE	0.034 (0.01)	56.7 (12)	2.96 (1.0)	0.83 (0.2)	3.72 (0.7)	340.16 (108.8)	40	21	19	0.525	0.959

to Hardy-Weinberg equilibrium was determined by assessing the significance of the F_{IS} values by means of Fisher's exact tests implemented in the GENEPOP v3.4 program, with specified Markov chain parameters of 5000 dememorization steps, followed by 1000 batches of 5000 iterations per batch. The sequential Bonferroni correction was applied to obtain critical confidence limits for multiple comparisons, with an initial a of 0.05 (Holms 1979).

To examine whether there was any accumulation of genetic diversity in downstream populations we tested for associations between genotypic (G, G_L, P and D) and genetic (A, H_E and H_O) parameters and the position of populations along the course of the river (expressed in meters from the most upstream to the most downstream population), by means of separate regression analyses.

Bayesian-based inference of population structure

We employed several methods to assess population structure. First, the genetic structure of the populations was examined with two fully Bayesian clustering methods: BAPS (Bayesian Analysis of Population Structure) version 3.1 (Corander *et al.* 2003, 2004) and STRUCTURE version 2.1 (Pritchard *et al.* 2000). BAPS v.3.1 estimates hidden population substructure by clustering populations

(*i.e.* geographical sampling locations) into panmictic groups (having a range of reasonable values of [1, N_p], with N_p representing the total number of geographical sampling locations), based on expected Hardy-Weinberg equilibrium and linkage equilibrium between loci within each of the observed populations. BAPS version 3.1 uses stochastic optimization, as opposed to the MCMC algorithm used in BAPS 2.0, to infer the posterior mode of the genetic structure (Corander *et al.* 2006). In addition we used STRUCTURE v.2.1 to obtain a separate insight into how the genetic variation is organized based on the clustering of individuals (rather than populations) without prior information on the population of origin. STRUCTURE v.2.1 uses a Bayesian MCMC approach to cluster individuals into *K* panmictic groups, by minimizing deviations from Hardy-Weinberg equilibrium and linkage equilibrium. The program calculates an estimate of the posterior probability of the data for a given *K*, Pr($X|K$) (Pritchard *et al.* 2000). In order to quantify the amount of variation of the likelihood for each *K* we performed a series of 10 independent runs for each value of *K*, with *K* ranging from 1 to the number of geographical sampling locations (N_p) plus one. We assumed an admixture model with correlated allele frequencies, using a length of the burn-in and MCMC iterations of 10 000 each. Longer burn-in and MCMC iterations did not significantly change the results. It has been shown that in many cases $Pr(X|K)$ may still increase slightly, even after the real *K* is reached (Pritchard & Wen 2004; Evanno *et al.* 2005), making inferences of *K* solely based on the highest values of $Pr(X|K)$ difficult. We therefore used Evanno *et al.*'s (2005) ad hoc statistic, ΔK, which is based on the second order rate of change of $Pr(X|K)$ with respect to *K* ($\Delta K = m(|L(K+1)-2L(K)+L(K-1)|)/s[L(K)]$). This ad hoc statistic ΔK should show a clear peak at the uppermost hierarchical level of structure at the true value of K (see Evanno *et al.* 2005, for a detailed description).

BAPS and STRUCTURE are fully Bayesian approaches, implicitly assuming that all true populations of origin have been sampled (Manel *et al.* 2002, 2005). As a result, they do not take into account that some individuals may originate (as a result of recent migration) from source locations outside the studied sampling area. To identify potential immigrants from outside our river systems we used Rannala & Mountain's (1997) partial exclusion Bayesian-based assignment method, implemented in GENECLASS version 2.0c (Piry *et al.* 2004), to compute the likelihood of each individual's genotype into each of the inferred clusters. To avoid possible bias as a result of 'self assignment' the 'leave one out' procedure was followed, which excludes the tested individual when calculating the allele frequency distribution of their own population. We used the Monte Carlo resampling method by Paetkau *et al.* (2004) implemented in the GENECLASS software, to generate a statistical threshold (using a number of simulated individuals of 10.000) beyond which individuals, whose multi-locus genotypes lie outside the 95% likelihood of a population, are likely to be excluded from that population, *i.e.* they were considered to be immigrants (Berry *et al.* 2004).

Isolation by distance

We followed the method proposed by Rousset (1997) to test the null hypothesis of a single migrant

pool over a whole river system against isolation by distance (IBD). Two different distance measures were used to estimate genetic distances among populations: First, traditional *F*-statistics were used to estimate $F_{ST}/(1- F_{ST})$ among populations according to Weir & Cockerham (1984), using FSTAT v. 2.9.3.2 (Goudet 1995); Second, Bayesian-based assignment procedures (Rannala & Mountain 1997) were used to calculate D_{LR}-distances among populations, using the program SPASSIGN (Pálsson 2004). D_{LR}-values (*i.e.* genotype likelihood ratio distances) represent the average orders of magnitude of the likelihood that the genotypes of individuals, of two populations being compared, are to occur in the individuals' own population, rather than in the other population (Paetkau *et al.* 1997). D_{LR}-values therewith represent an assignment-based measure of distance among populations (Pálsson 2004). A Mantel test was used to test for the presence of isolation by distance, using FSTAT v. 2.9.3.2 (Goudet 1995).

Results

Field survey

We found significant differences in plant density, plant morphology and plant biomass within *S. emersum* populations between the Swalm and Rur rivers (Table 1). In the river Swalm plant populations had a significantly higher mean (±SD) plant density compared to plant populations in the river Rur (278.15±68.8 and 100.28±24.1 plants $\cdot m^{-2}$, respectively; df=1, F=64.71, P<0.001). We also observed differences in plant morphology between rivers: In the river Swalm, only submerged plants were found (*i.e.* with very fragile, thin, and flexible ribbon-formed leaves), whereas in the river Rur, both submerged and emergent plants were observed (*i.e.* the latter having sturdy, erect, emergent leaves and often a thick flowering stem). This difference in plant morphology was expressed in observed differences in plant biomass between rivers, with significantly lower plant biomass (dry weight) found in populations of the river Swalm, compared to the river Rur (0.41±0.1 and 2.04±0.7 g $\cdot plant^{-1}$, respectively; df=1, F=74.49, P<0.001). These differences in plant density, biomass and morphology coincided with an approximately 10-fold higher stream velocity within plant populations in the river Swalm compared to the river Rur (Table 1).

The differences in plant morphology also reached expression in observed differences in sexual reproduction between rivers, as assessed by the proportion of flowering plants and the seed production per plant. Notably, sexual reproduction was not observed in any of the populations in the river Swalm, whereas it was observed in all, but one (population RAT), of the populations in the river Rur (Table 1).

Genetic and genotypic diversity

The total number of alleles observed per locus in the overall sample of 606 individuals ranged from 8 (SEM14) to 17 (SEM05), with an overall total of 77 alleles scored over 6 loci (Appendix 1). Significant departures from Hardy-Weinberg equilibrium were observed in 21 of the 60 single-locus

exact tests after sequential Bonferroni correction (populations with $N \leq 2$ genets not considered; Appendix 1). There was no evidence for linkage between any of the pairs of loci. Negative overall F_{IS} values were observed in all populations, however, a Hardy-Weinberg global test for heterozygote excess on F_{IS} values across loci revealed a significant heterozygote excess for only two populations in the River Rur (RAT and ORS; $P< 0.05$).

The theoretical number of possible genotypes (N_g), with the six loci used, was 3.26×10^{11}. The P_{gen} values for each multilocus genotype ranged from 1.33×10^{-17} to 4.72×10^{-4}. Since, the P_{gen} values did not exceed the threshold of 0.001 for any given genotype, the microsatellite loci used in this study allowed the unequivocal assignment of ramets to clones. There was a large difference in genotypic diversity between the two rivers (Table 1). Compared to plant populations in the Rur river, populations in the Swalm river displayed a significantly lower mean (±SD) number of genotypes G (27.5±12 and 3.9±5, respectively; Mann-Whitney U test, U=5.500, P=0.003), number of local genotypes G_L (23.6±10 and 3.1±6; U=2.500, P=0.001), proportion of distinguishable genotypes P (0.70±0.3 and 0.11±0.1; U=3.000, P=0.001) and Simpsons' diversity index D (0.87±0.3 and 0.20±0.3; U=5.500, P=0.003). Almost all populations in the Rur river consisted of a large number of genotypes, most of which were unique for that population (Table 1). Of the 248 multilocus genotypes that were found in the Rur river, only 10 occurred in more than one population. These ramet-pairs with identical multilocus genotypes were not restricted to neighbouring populations, but were randomly found between population pairs (regardless of their proximity to eachother). In contrast, the populations in the river Swalm were either monoclonal or consisted of a few genotypes only. Moreover, a clear spatial separation of genotypes was observed in the Swalm river: The five populations in the river Swalm situated upstream of Lake Hariksee were dominated by a single genotype, while the three populations situated downstream of Lake Hariksee were also dominated by a single, though different, genotype. Only in the two populations lying at the upstream and downstream edge of Lake Hariksee (BRE and BRU, respectively), a few other genotypes were found (Table 1).

Regression analyses did not reveal any significant associations between genetic (A, H_E and H_O) or genotypic (G, G_L, P and D) parameters and the position of populations along the course of either the Rur or Swalm rivers ($P > 0.05$ for all regressions), indicating that there was no accumulation of genetic diversity in downstream populations.

Bayesian inference of population structure

The BAPS (Corander *et al.*, 2004) analysis, which used the geographical information given by the sampling location, revealed a strong optimal partitioning of the 17 populations into three clusters (Table 2): cluster 1, consisting of all nine populations of the Rur river; cluster 2, consisting of populations one to five of the Swalm river; and cluster 3, consisting of populations six to eight of the Swalm river. The absolute values of changes in the logarithm of the marginal likelihoods (logml) ranged from 25 to 365 (much larger then the threshold value of 2.3 given by Corander & Marttinen 2005), indicating that the optimal partitioning into these three groups was very stable

Table 2 Population structure of the 17 *S. emersum* populations (Rur and Swalm rivers), inferred from the BAPS analyses. Given are the goodness-of-fit levels, in terms of changes in the natural logarithm of the marginal likelihood of the data (logml-values) if group *i* is moved to cluster *j*, for the optimal clustering solution of BAPS (Corander & Marttinen 2005).

		Inferred population clusters		
River	Population	1	2	3
Rur	RUR	0	-25.2	-35.5
	LIN	0	-184.5	-319.2
	BRA	0	-222.8	-365.0
	TEN	0	-228.2	-338.9
	HIL	0	-126.8	-242.6
	RAT	0	-175.4	-314.2
	ORS	0	-198.2	-318.2
	MEL	0	-191.9	-353.5
	ROE	0	-160.5	-255.8
Swalm	LOC	-46.1	0	-35.6
	GEN	-77.0	0	-46.8
	LUT	-46.1	0	-35.6
	PAN	-46.1	0	-35.6
	BRE	-209.5	0	-145.2
	BRU	-485.2	-164.6	0
	ZWE	-63.2	-37.6	0
	HOO	-63.2	-37.6	0

Table 3 The proportion of individuals from each sample location assigned to each of the clusters (K) inferred from the STRUCTURE analysis, for each river separately. Proportions greater than 0.5 are shown in bold. (N_{gen} = the number of genets in each population; see Table 1).

Inferred population clusters Rur				Inferred population clusters Swalm				
Population	N_{gen}	1	2	Population	N_{gen}	1	2	3
RUR	1	0.013	**0.987**	LOC	1	**0.992**	0.004	0.003
LIN	37	0.477	**0.523**	GEN	2	**0.981**	0.004	0.015
BRA	36	**0.543**	0.457	LUT	1	**0.993**	0.004	0.004
TEN	38	0.500	0.500	PAN	1	**0.994**	0.003	0.003
HIL	21	0.355	**0.645**	BRE	11	**0.560**	0.430	0.009
RAT	32	**0.657**	0.343	BRU	13	0.012	0.005	**0.983**
ORS	30	0.470	**0.530**	ZWE	1	0.006	0.005	**0.989**
MEL	32	**0.607**	0.393	HOO	1	0.008	0.004	**0.988**
ROE	21	0.418	**0.582**					

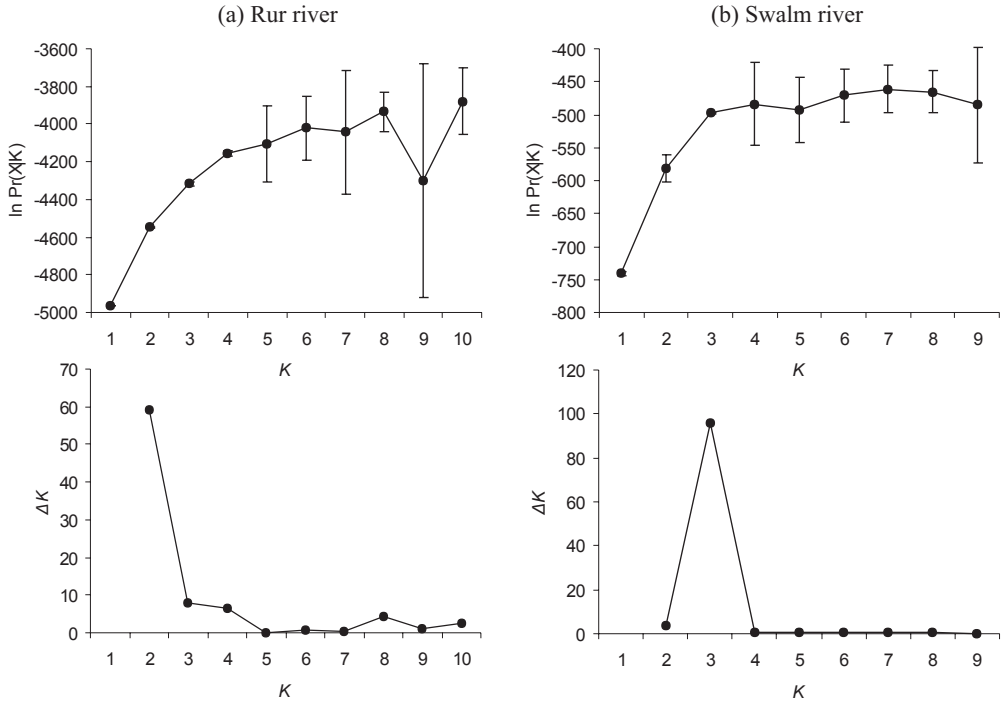

Fig 2 Results of Bayesian clustering (STRUCTURE, Pritchard *et al.* 2000) of *S. emersum* individuals from (a) the Rur and (b) the Swalm rivers. The upper graphs give the mean ln $Pr(X|K)$ (± SD) over 10 runs for each value of *K*. The lower graphs give Evanno *et al.*'s (2005) ad hoc statistic ΔK, showing a peak at the uppermost level of structure at the true value of *K*.

(Table 2). When analysing the data of the two rivers pooled together, the STRUCTURE (Pritchard *et al.*, 2000) analysis could not infer an optimal structuring into *K* populations: Ln(*K*) kept increasing with increasing *K*, even at $K>N_p$, and no clear peak was found after applying Evanno *et al.*'s (2005) posterior ⊿*K* statistic. However, when the number of *K* populations was estimated for each river separately, the results were very consistent with the outcome of BAPS. For the 9 populations of the river Rur an optimal partitioning of $K\leq2$ clusters was found (Figure 2a). The results in Table 3 show that for $K=2$, the populations are roughly symmetrically assigned to the two clusters. The results are therefore more in favour of considering the populations of the river Rur as one single population, *i.e.*, $K=1$, rather than two separate clusters (Pritchard & Wen 2003). For the 8 populations of the river Swalm an optimal optimal partitioning of $K=3$ cluster was found (Figures 2b). However, when viewing the proportions of individuals greater than 0.5 assigned to each of the three clusters (Table 3), the analysis seems more in favour of two distinct clusters: populations 1 to 5 and populations 6 to 8 (Table 3).

The results of both the BAPS and STRUCTURE analyses therefore support a partitioning of the 17 populations into three distinct clusters: cluster 1 (population 1-9 of the Rur), cluster 2 (population 1-5 of the Swalm) and cluster 3 (population 6-8 of the Swalm). The GENECLASS analysis identified 7 individuals that could not be assigned to any of the 3 clusters (3 individuals from cluster 1, 2 from cluster 2, and 2 from cluster 3), indicating recent immigration events from

Table 4 The proportion of *S. emersum* individuals assigned to each of the three clusters using GENECLASS2.0 (Piry *et al.* 2004). NA represents the proportion of individuals that was not assignable to any of the three clusters ($P < 0.05$).

	C1	C2	C3	NA
C1	0.984	0	0	0.016
C2	0	0.875	0	0.125
C3	0	0	0.933	0.067

sources outside our study area (Table 4).

Mantel tests did not reveal a significant relationship between geographical and genetic distances among populations of the river Rur, for either the Bayesian-based D_{LR} distances (r=0.157, P=0.4365) or the $F_{ST}/(1- F_{ST})$ distances (r=0.242, P=0.225). The low F_{ST}-values among populations in the Rur (ranging from -0.0009 to 0.0577) and the absence of isolation by distance, concur with the conclusion inferred from the BAPS and STRUCTURE analyses, that the nine populations of the Rur should be viewed as a single population.

Discussion

Genotypic diversity within populations in relation to mode of reproduction

In riverine habitats, hydraulic forces from water currents may have a large impact on plant morphology. Riparian plants respond to increasing water velocity through plastic morphological changes in order to reduce mechanical damage (*e.g.* reduction of plant size and biomass, decreased spacerlength leading to higher plant density, increased stem and leaf flexibility reducing rigidity and frontal area) (Chambers *et al.* 1991; Schutten & Davy 2000; Boeger & Poulson 2003; Puijalon & Bornette 2004; Puijalon *et al.* 2005).

Likewise, *S. emersum* will, when subjected to different hydrodynamic conditions, form plants with different morphologies: totally submerged plants in high velocity areas and emergent plants in slow flowing areas (Haslam 1978; Ságová-Marečková & Květ 2002). In the Swalm river, characterized by an approximately 10-fold higher flow velocity compared to the Rur river, only submerged plants were observed, displaying typical morphological adaptations to withstand the associated pulling forces of the water; *i.e.* reduced plant size and above ground biomass and increased plant density (resulting in a more compact growth form reducing forces on individual ramets) and short, thin and flexible leaves (reducing drag stress; Sand-Jensen 1998). These morphological differences have consequences for the plants' ability for sexual reproduction (Haslam 1978; Boeger & Poulson 2003); since *S. emersum* relies on wind-mediated pollen dispersal, submerged plants are not capable of sexual reproduction.

This difference in the mode of reproduction between *S. emersum* populations of the Swalm and Rur rivers, corresponds to a remarkable difference in genotypic diversity. In the Swalm river the high water velocities induce morphological adaptations that prevent plants from emerging from

the water, limiting their ability for sexual reproduction, and ultimately leading to low genotypic diversity within *S. emersum* populations. Whereas, in the Rur river the occurrence of low-velocity patches allows plants to emerge from the water and reproduce sexually, effectively leading to high genotypic diversity within *S. emersum* populations.

Regional population structure in the Rur river

The local mode of reproduction also has an impact on the regional dispersal processes. In the Rur river, where populations were reproducing sexually, the Bayesian-based inference of population structure as well as the low pair-wise genetic distances (F_{ST}-values), indicate little genetic differentiation among the nine *S. emersum* populations. The results strongly suggest that the nine populations of the Rur river should be viewed as a single population, with high levels of gene flow occuring between them, in spite of large distances (up to 50 km). The high levels of gene flow most likely arise from hydrochoric dispersal of generative propagules (seeds) between the *S. emersum* populations: (i) Sexual reproduction was observed in all (but one) of the studied populations (this study); (ii) seed buoyancy experiments have shown that *S. emersum* plants produce long-floating seeds (floating durations ranging from a few days up to several months; Pollux unpublished); and (iii) germination experiments have shown that seeds remain viable regardless of the duration of their buoyancy (Pollux unpublished).

Of the 248 genotypes found in the Rur river, only 10 were found in more than one population. This spatial separation of ramets indicates dispersal between populations by means of vegetative propagules (Nilsson *et al.* 1991; Boedeltje *et al.* 2004). The detection of a small number of identical genotypes, however, suggests that dispersal of vegetative propagules is a relatively rare event for *S. emersum* in the Rur river (Kitamoto *et al.* 2005).

Regression analyses of genetic and genotypic diversity parameters within populations against the position of *S. emersum* populations along the Rur river, did not reveal any significant relationships, indicating that there was no accumulation of diversity towards downstream located *S. emersum* populations. Although such associations have been found in a few studies, *e.g.* in *Potamogeton coloratus* (Gordano valley, UK), *Angelica archangelica* (Vindel river, Sweden) and *Myricaria laxiflora* (Yangtze river, China) (Gornall *et al.* 1998; Lundqvist & Andersson 2001; Liu *et al.* 2006), most studies failed to reveal any effect of unidirectional gene flow on the pattern of genetic variation along rivers, *e.g.* in *Mimulus caespitosus* (mountain streams, Washington, USA), *Calycophyllum spruceanum* (Amazon Basin, Peru), *Bistorta vivipara* and *Viscaria alpina* (Vindel river, Sweden), *Populus nigra* (Drôme river, France), *Silene tatarica* (Oulankajoki river, Finland) or *Helmholtzia glaberrima* (Toolona creek, Australia) (Ritland 1989; Russel *et al.* 1999; Lundqvist & Andersson 2001; Imbert & Lefèvre 2003; Tero *et al.* 2003; Prentis *et al.* 2004). This lack of genetic erosion in upstream areas may be related to dispersal in an upstream direction, either by means of wind-mediated pollen dispersal or animal-mediated seed dispersal, resulting in the introduction of alleles from downstream to upstream areas (Pollux *et al.* 2005). Several genetic studies have provided evidence for waterfowl-mediated seed dispersal in aquatic plant species (Mader *et al.* 1998; King *et al.* 2002), and a few studies have provided evidence

for the occurrence of upstream dispersal events in river systems (Tero *et al.* 2003; Imbert & Lefèvre 2003).

Regional population structure in the Swalm river

The spatial distribution of genotypes, as well as the Bayesian-based inference of population structure, suggest that the eight populations of the Swalm river were (i) monoclonal or dominated by a few genotypes only, and (ii) divided in two independent genetic neighbourhoods, separated by Lake Hariksee.

Two contrasting hypotheses, that might explain the emergence of such a population structure are conceivable. Firstly, the six monoclonal populations (together comprising only 3 genotypes) in the Swalm river may have originated from introductions of a very few individuals to the upper and lower reaches of the Swalm river, which were then followed by local clonal growth. Moreover, plant fragments of *S. emersum* are positively buoyant and have highly regenerative abilities (Barrat-Segretain *et al.* 1998, 1999) and although hydrochoric dispersal of clonal plant fragments may be a relatively infrequent mechanism of dispersal for *S. emersum* (see above; Boedeltje *et al.* 2004), it may, in the absence of seed dispersal, still lead to succesfull colonisation of suitable habitat patches (Barrett *et al.* 1993; Kitamoto *et al.* 2005). The hydrochoric dispersal of clonal plant fragments, therefore, offers a likely explanation why several of the discrete monoclonal populations in the Swalm river, situated (tens of) kilometers apart, consisted of the same genotype (*i.e.* populations 1 to 5 and 6 to 8, respectively). Secondly, the populations in the Swalm river may originally have consisted of genotypically diverse populations. In a prolonged absence of sexual reproduction (due to a suppression by environmental conditions, see above), genetic processes, such as genetic drift and selection, may subsequently have resulted in the broad dominance of best-fitted 'single-purpose genotypes' (sensu Barrett *et al.* 1993; Honnay & Bossuyt 2005). Less adapted clones may have become outcompeted by ramets of more adapted genotypes, ultimately leading to monoclonal populations (Honnay & Bossuyt 2005). However, we found no evidence that, in the past, the hydrological regime in the Swalm river would have allowed sexual reproduction of *S. emersum*, potentially arguing against hypothesis 2. Unfortunately, as historical information about the genetic structure of populations in the Swalm river is not available, we are unable to reliably state which of the two proposed hypotheses is most likely true.

Dispersal between river systems

In both the Rur and Swalm rivers, the GENECLASS analysis revealed a total of 7 possible immigrants originating from outside our study area. These immigrants most likely originated from nearby lowland rivers and streams, where *S. emersum* is a common species. Although the vector of dispersal remains unknown, it is known that (i) in these lowland rivers and streams many waterfowl species are, during fall and winter, feeding on seeds of aquatic plants (*e.g. Sparganium* spp.), and that (ii) ingested *S. emersum* seeds can be internally transported by waterfowl, while remaining viable after

gut passage (Pollux *et al.* 2005 and references therein). We therefore suggest that waterfowl-mediated seed dispersal is the most likely vector for plant dispersal between different river systems.

General Conclusions

This study shows that spatial heterogeneity in the hydrodynamic regime may induce local differences in the mode of reproduction (sexual vs asexual) in riparian plant species (*e.g. S. emersum*, *Sagittaria sagittifolia*, *Berula erecta*, *Veronica anagallis-aquatica*; Haslam 1978; Ságová-Marečková & Květ 2002; Boeger & Poulson 2003; Puijalon unpublished), affecting both the clonal structure and genetic diversity within populations, as well as the regional population structure. The outcome of this study, furthermore, shows that the clonal structure and dispersal processes of riparian plants may differ greatly between river systems, depending on differences in environmental conditions between rivers (see also Kitamoto *et al.* 2005).

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Appendix 1 Number of alleles (*A*), expected (*HE*) and observed (*HO*) heterozygosities, and deviations from HWE (F_{IS}) according to Weir & Cockerham (1984). Values in bold indicate samples which deviate significantly from HWE (P<0.05) after sequential Bonferroni corrections. All calculations are based on genet-level analyses (Ngen = sample size, *i.e.* the number of distinguishable genets; note that several populations consist of only one genet; see Table 1).

		Swalm River								Rur River								
		LOC	GEN	LUT	PAN	BRE	BRU	ZWE	HOO	RUR	LIN	BRA	TEN	HIL	RAT	ORS	MEL	ROE	
Locus	N_{Gen}	1	2	1	1	11	13	1	1	1	37	36	38	21	32	30	32	21	A_{Tot}
SEM01	A	2	2	2	2	3	6	2	2	2	7	5	5	3	4	6	4	4	11
	HE	1.0000	0.6667	1.0000	1.0000	0.6474	0.8308	1.0000	1.0000	1.0000	0.6467	0.6786	0.5407	0.5528	0.5352	0.5717	0.4981	0.7094	
	HO	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.6053	0.7568	0.6316	0.4762	0.7500	0.7097	0.6857	0.8800	
	FIS	-	-	-	-	-0.593	-0.214	-	-	-	0.054	-0.109	-0.171	0.142	-0.390	-0.246	-0.391	**-0.229**	
SEM05	A	2	2	2	2	4	3	2	2	2	6	9	12	7	8	8	9	8	17
	HE	1.0000	0.6667	1.0000	1.0000	0.7835	0.6431	1.0000	1.0000	1.0000	0.6774	0.7653	0.7969	0.7026	0.7184	0.7097	0.8334	0.7967	
	HO	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.8286	0.9444	0.9355	1.0000	1.0000	1.0000	1.0000	0.9524	
	FIS	-	-	-	-	**-0.294**	-0.592	-	-	-	-0.213	**-0.247**	-0.177	-0.425	**-0.396**	**-0.421**	-0.199	-0.174	
SEM08	A	2	2	2	2	5	3	2	2	2	8	10	11	8	7	8	9	8	16
	HE	1.0000	0.6667	1.0000	1.0000	0.8235	0.6769	1.0000	1.0000	1.0000	0.8477	0.8736	0.8641	0.7944	0.8131	0.8519	0.8396	0.8670	
	HO	1.0000	1.0000	1.0000	1.0000	0.8889	0.7692	1.0000	1.0000	1.0000	0.9211	0.9412	1.0000	1.0000	0.9091	0.9643	0.8788	0.9583	
	FIS	-	-	-	-	**-0.085**	**-0.143**	-	-	-	**-0.085**	**-0.075**	**-0.160**	**-0.239**	**-0.133**	-0.135	**-0.078**	**-0.086**	
SEM12	A	2	3	2	2	3	3	1	1	2	5	4	5	3	4	4	5	4	10
	HE	1.0000	0.8333	1.0000	1.0000	0.5750	0.6615	0.0000	0.0000	1.0000	0.3347	0.4532	0.3446	0.1991	0.4344	0.3435	0.2691	0.6082	
	HO	1.0000	0.5000	1.0000	1.0000	0.7500	0.0769	0.0000	0.0000	1.0000	0.3784	0.5405	0.3421	0.1053	0.5357	0.3929	0.2941	0.7917	
	FIS	-	-	-	-	-0.333	**0.888**	-	-	-	-0.137	-0.162	0.007	0.477	-0.239	-0.147	-0.085	-0.265	
SEM14	A	2	2	2	2	3	7	2	2	2	5	5	6	3	3	4	5	4	8
	HE	1.0000	0.6667	1.0000	1.0000	0.6474	0.8431	1.0000	1.0000	1.0000	0.6774	0.6513	0.6414	0.5220	0.5499	0.5717	0.5834	0.6910	
	HO	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.8286	0.9630	0.8000	0.8421	0.9310	0.7931	0.8824	0.8500	
	FIS	-	-	-	-	-0.593	-0.195	-	-	-	0.110	-0.488	-0.252	-0.624	**-0.714**	-0.397	-0.539	-0.220	
SEM15	A	2	2	2	2	4	4	1	1	1	5	8	7	5	5	6	5	4	15
	HE	1.0000	0.6667	1.0000	1.0000	0.7273	0.4831	0.0000	0.0000	0.0000	0.7031	0.7559	0.7419	0.6307	0.6769	0.6732	0.7669	0.6738	
	HO	1.0000	1.0000	1.0000	1.0000	0.7273	0.5385	0.0000	0.0000	0.0000	0.9167	0.7778	0.9189	1.0000	1.0000	0.9677	0.8000	0.5833	
	FIS	-	-	-	-	0.0000	-0.120	-	-	-	-0.308	**-0.093**	-0.243	**-0.573**	**-0.497**	**-0.448**	**-0.076**	**0.022**	
Overall	HE	1.0000	0.6905	1.0000	1.0000	0.7274	0.5912	0.5714	0.5714	0.7143	0.6597	0.7017	0.6649	0.6006	0.6323	0.6291	0.6503	0.7087	
	HO	1.0000	0.9286	1.0000	1.0000	0.8705	0.6264	0.5714	0.5714	0.7143	0.6946	0.7729	0.7405	0.7272	0.8483	0.8087	0.7411	0.7908	
	FIS	-	-	-	-	-0.196	-0.060	-	-	-	-0.042	-0.116	-0.116	-0.200	-0.353	**-0.292**	**-0.158**	-0.119	

Chapter 7

Assessing the regional population structure of the aquatic macrophyte *Sparganium emersum*: spatially extended population, metapopulation or regional ensemble?

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(submitted)

Summary

1. *Regional populations of plants may be classified as spatially extended populations, regional ensembles or metapopulations, depending on the importance of local versus regional dynamics. However, the ascription of plant populations to one of these three groups, based solely on field observations, may be very difficult due to a number of plant-specific problems associated with the metapopulation concept.*

2. *The use of molecular markers in a population genetic approach may help to reveal the regional structure of plant populations. We propose a number of testable hypotheses about the genetic structure of populations and the rate of gene flow between the populations, for each of the three groups, which may be used to distinguish the three groups.*

3. *We used these testable hypotheses to examine the regional population structure of the unbranched burreed (Sparganium emersum) along the Niers River (Germany - the Netherlands), using Amplified Fragment Length Polymorphisms (AFLP).*

4. *The results revealed a clear genetic population differentiation, highly variable Φ_{ST} values, an absence of isolation by distance, and the occurrence of gene flow between populations. The analyses furthermore revealed an asymmetry in the direction of gene flow, with gene flow occurring predominantly in a downstream direction. In accordance, the genetic diversity within populations increased from upstream to downstream located populations along the Niers River.*

5. *The pronounced genetic differentiation among the S. emersum populations argued against the existence of a single panmictic spatially extended population, while the inference of dispersal events among populations argued against the presence of a regional ensemble. The genetic population structure and rate of gene flow were therefore most in agreement with the metapopulation model.*

6. *The results of this study suggest that population genetic analyses may prove to be a helpful tool when assessing the regional population structure.*

Introduction

Dispersal is a fundamental process in population genetics and (meta)population ecology (Ouborg *et al.* 1999; Ouborg & Eriksson 2004). The quantification of plant dispersal is notoriously difficult and several different approaches have been used in the past: (i) Empirical approaches, which measure dispersal by trapping seeds or seed mimics at various distances from the source plants (Nilsson *et al.* 1991; Craddock & Huenneke 1997; Boedeltje *et al.* 2004); (ii) Mechanistic approaches, which test the dispersal capacity of seeds under controlled conditions and relate this information to the 'behaviour' of the putative dispersal vectors (wind, water, animals) to predict potential dispersal distances (Nathan *et al.* 2002; Soons *et al.* 2004); and (iii) Molecular approaches, which use the distribution of genetic variation within and among populations to make inferences about the rate of gene flow that has occurred between them (Tero *et al.* 2003; He *et al.* 2004). It has been argued that molecular approaches are very useful for studying plant dispersal for two reasons: First, because molecular approaches are capable of distinguishing between gene flow resulting from the dispersal of generative (seeds) and vegetative (clonal plant fragments) propagules; and second, because rare, though often biologically relevant, dispersal events (*e.g.* long-distance dispersal) are likely to be underestimated when using empirical and mechanistic approaches (Ouborg *et al.* 1999). In particular, assignment tests form promising and popular new statistical tools for inferring dispersal on ecological time-scales (Manel *et al.* 2002, Berry *et al.* 2004; Paetkau *et al.* 2004; Manel 2005).

The application of the metapopulation concept to questions concerning the spatial structure of plant populations is under debate (Eriksson 1996; Husband & Barrett 1996; Bullock *et al.* 2002; Freckleton & Watkinson 2002, 2003; Ehrlén & Eriksson 2003; Pannell & Obbard 2003; Ouborg & Eriksson 2004; Murphy & Lovett-Doust 2004). Freckleton & Watkinson (2002) argued that, based on the spatial arrangement of suitable habitats and the importance of local (births, deaths) *vs* regional dynamics (extinction, recolonization), a set of local plant populations at regional scales can be classified into either of three groups: (1) '*Spatially extended populations*', which occur as clumps or patches of a single population in a large continuous area of suitable habitat. Spatially extended populations are dominated by local processes, in which patchiness arises as a consequence of local dispersal and local disturbances. (2) '*Regional ensembles*', which consist of a regional set of highly persistent (*i.e.* little or no local extinction) and basically unconnected (*i.e.* no migration) populations. The sizes and persistence populations are entirely determined by local processes. (3) '*Metapopulations*', which exist as a series of local populations, in which regional processes (population extinction and recolonization) dominate and suitable habitat occurs as discrete patches within a larger matrix of unsuitable habitat.

In the field, the ascription of populations to any of these three groups may be very difficult due to a number of plant-specific problems associated with the metapopulation concept (Freckleton & Watkinson 2002; Ouborg & Eriksson 2004). In this paper, we assess to what extent genetic analyses may be helpful in revealing the regional structure of plant populations and estimating the rate of dispersal between them. To this end, we formulate a number of theoretical predictions about the genetic structure and the rate of gene flow, for each of the three models described by Freckleton & Watkinson (2002).

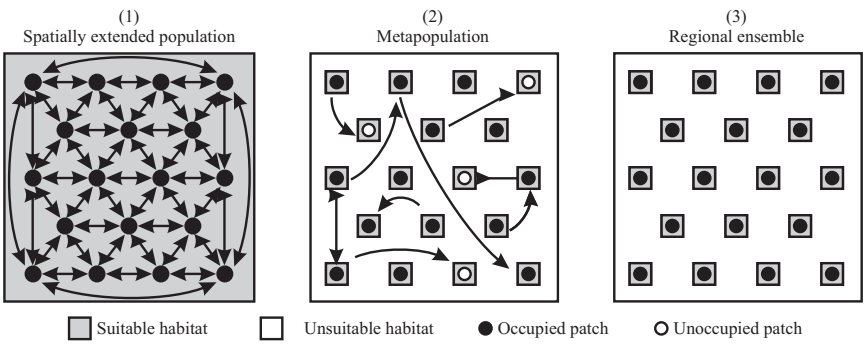
Hypotheses

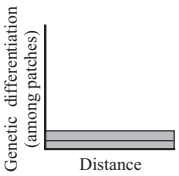
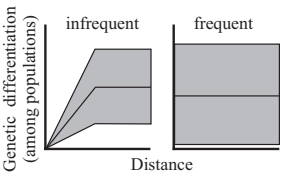
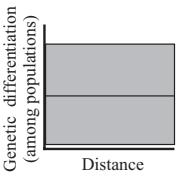
In a spatially extended population (Freckleton & Watkinson 2002; Table 1), the plant patches are expected to form a single genetically uniform panmictic unit with free gene flow between them (Tero et al. 2003). Since, local dispersal (among patches within the spatially extended population) is much more important than drift, measures of pairwise patch differentiation (F_{ST} or Φ_{ST}) may be expected to be low and non-significant, and should not increase with geographic distance (Hutchison & Templeton 1999; Koizumi et al. 2006). Moreover, since there is a direct positive relationship between the genetic differentiation between populations and the reliability of dispersal events between patches inferred from assignment tests (Waser & Strobeck 1998), assignment-based estimates of contemporary gene flow will show a high proportion of individuals that can not be reliably assigned to a single source patch (i.e. ambiguously assigned individuals; see Material & Methods). Hence, in spatially extended populations, the low pairwise genetic differentiation among patches will not yield reliable estimates of dispersal between patches.

In Metapopulations (Freckleton & Watkinson 2002; Table 1), drift and migration are both important. Assignment-based estimates of contemporary gene flow should reveal distinct dispersal events between populations (Tero et al. 2003; He et al. 2004). Moreover, population differentiation may be highly variable, because the dynamics of extinction and recolonization may lead to the formation of an age structure in which drift and migration have opposing effects on the genetic differentiation: i.e. newly founded populations will be highly differentiated from the other populations (since founding events represent a source of genetic drift), while this degree of population differentiation will decrease as the populations grow older (due to continued immigration from the other surrounding populations; Whitlock & McCauley 1990;Giles & Goudet 1997; Pannell & Charlesworth 2000). Whether or not the genetic distances between populations will increase with geographical distance between them will depend on frequency of extinction and recolonization events (Koizumi et al. 2006). If extinction and recolonization occur relatively infrequently, population differentiation is expected to increase linearly with increasing distance (in one-dimensional habitats) or log(distance) in two-dimensional habitats (Wright 1943; Rousset 1997; Koizumi et al. 2006), though it will level of at larger distances (Hutchison & Templeton 1999). However, if extinction and recolonization occur more frequently population differentiation will be highly variable due to the formation of an age structure, and a correlation between genetic and geographic distance will not be present (Koizumi et al. 2006; Jacquemyn et al. 2006).

In *regional ensembles* (Freckleton & Watkinson 2002; Table 1), drift is much more important than migration. Assignment-based estimates of gene flow should reveal a complete absence of contemporary dispersal between populations (Tero *et al.* 2003). Moreover, the isolated subpopulations are expected to show a strong population differentiation. In such isolated populations, the pairwise genetic distances among populations will not show any relationship with geographic distances (Hutchison & Templeton 1999; Koizumi *et al.* 2006) (note that, although the relationship between genetic and geographic distance of a regional ensemble may be comparable to that of a metapopulation with frequent turn over, they are both the result of fundamentally different processes; Table 1).

Table 1 Classification of regional plant population structures according to Freckleton & Watkinson (2002), and the theoretical predictions on the genetic structure and rate of gene flow between subpopulations (see main text for further details).



Definition and characteristics (according to Freckleton & Watkinson 2002)			
Definition	A population that exists in a large continuous area of habitat, dominated by local processes (i.e. births, deaths, within population dispersal)	A series of local populations, characterized by local extinctions, recolonizations and interpopulation migration.	A series of highly persistent and isolated (i.e unconnected) populations Extinctions and recolonizations are very rare to non-existent.
Habitat	Continuous suitable habitat	Discrete suitable habitat patches	Discrete suitable habitat patches
Importance of local dynamics (births, deaths, within population dispersal)	High	Low	High
Importance of regional dynamics	Low	High	Low
- Population extinction	Nil	Frequent	Rare - Nil
- Interpopulation migration	Nil	Common	Rare - Nil
- Recolonization	Nil	Common	Nil
Theoretical predictions of the genetic structure and the level of gene flow			
Relative importance of drift and gene flow	Drift << Gene flow Gene flow (among patches within the spatially extended population) is more important than drift	Drift = Gene flow Drift and gene flow among populations are both important	Drift >> Gene flow Drift is more important than gene flow among populations
Relationship between pairwise genetic and geographic distance (F_{ST} *vs* geograhic distance)	Genetic differentiation will be low and non-significant, even among distant populations (i.e. the anlyses will reveal a single genetically uniform panmictic population). There will be no correlation between genetic and geographic distance, i.e. no IBD (Hutchison & Templeton 1999; Koizumi *et al.* 2006).	The relationship between genetic and geographic distance depends on the frequency of extinction and colonization events: If extinction and recolonization occurs less frequently and gene flow is more effective at shorter distances, there will be a pattern of isolation by distance, which may level off at larger distances (Hutchison & Templeton 1999). If extinction and recolonization occur more frequently, the genetic differentiation will be highly variable, and there will be no isolation by distance (Koizumi *et al.* 2006).	Genetic differentiation among populations will be highly variable due to both, the complete absence of gene flow and the occurrence of population bottlenecks. There will be no correlation between genetic and geographic distance, i.e. no IBD (Hutchison & Templeton 1999; Koizumi *et al.* 2006).
			
Gene flow (inferred from populations assignment tests)			
- Unambiguous assignment to population of origin	None	Most	All
- Unambiguous assignment to another population	None	Few	None
- Ambiguous assignments	All	Few	None
- Degree of gene flow	Inferrence of gene flow is not possible, due to the large proportion of ambiguous assignments	Little gene flow between the populations	No gene flow between the populations

In this study, we employed Amplified Fragment Length Polymorphisms (AFLP; Vos *et al.* 1995) to examine the regional population structure of the unbranched burreed (*Sparganium emersum*, Rehmann 1871) along the Niers River (Germany - the Netherlands). In particular, we examined (i) the extent and patterns of genetic variability within and among the *S. emersum* populations in the Niers River, (ii) the degree of population differentiation, (iii) the relationship between genetic and geographic distances among populations, and (iv) the rate and the direction (upstream *vs* downstream) of gene flow between populations. We then used this information to infer the spatial structure of plant populations, i.e. spatially extended population, regional ensemble or metapopulation structure (sensu Freckleton & Watkinson 2002), based on the theoretical predictions presented above.

Material & methods

Study species

Unbranched burreed, *S. emersum* Rehmann 1871 (= *S. simplex* Hudson 1778) (Sparganiaceae), is an aquatic vascular macrophyte, that is widely distributed throughout Eurasia and North America (Cook & Nicholls 1986). It typically grows in a narrow band at the margins of rivers, streams and canals that are characterized by shallow, slow flowing and nutrient-rich waters. *S. emersum* is a monoecious species, with temporally separated male and female flowers (pollen release preceding sigma receptivity; Sargent & Otto 2004). *S. emersum* flowers from June to August, and its flowers are mainly wind-pollinated (Sargent & Otto 2004). The seeds are released in autumn and are mainly dispersed by water currents and aquatic animals (Boedeltje *et al.* 2004; Pollux *et al.* 2005, 2006). Vegetative plant fragments are also dispersed by water currents; they remain viable and capable of establishment even after floating for up to 10 weeks (Barrat-Segretain & Amoros 1996; Barrat-Segretain *et al.* 1998, 1999). *S. emersum* is also capable of asexual (clonal) reproduction through the production of stolons, from which new ramets emerge (Cook & Nicholls 1986; Pollux *et al.* 2007). All above-ground biomass disappears during the winter and plants regenerate from underground rhizomes in spring (Wiggers-Nielsen *et al.* 1985; Greulich & Bornette 2003).

Study site

The Niers River (catchment surface area of 1348 km^2) originates near Kuckum (Erkelenz, close to Mönchengladbach, Germany), flows through Germany (106 km) and The Netherlands (8 km) where it discharges in the Meuse River (near Gennep, the Netherlands). In the fall of 2004, a total of 283 ramets of *S. emersum* were collected at nine locations along the Niers River (Germany – the Netherlands). In each location, plants were collected at 1 to 2 meter intervals along a linear transects running parallel to the shore. Plant samples were immediately transferred to 1.5 ml eppendorf tubes and stored at -80°C until DNA extraction. Genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen Genomics Inc.).

DNA isolation and AFLP protocol

AFLP analyses were performed according to Vos *et al.* (1995) with minor modifications (described below). Genomic DNA was digested in a total volume of 40µl (containing 10µl gDNA isolate, 8µl 5xR/L-buffer , 0.5µl *Eco*RI (10U/µl; Fermentas), 0.5µl *Mse*I (10U/µl; Fermentas) and 21µl milliQ) placed at 37°C for 1 hour. Adapters were ligated by adding a total volume of 10µl (containing 2µl 5xR/L-buffer, 1µl *Eco*RI-adapter (5pmol/µl), 1µl *Mse*I-adapter (50pmol/µl), 0.2µl T4-DNA-ligase (1U/µl; Fermentas), 1µl ATP (10mM, pH 7.0) and 4.8µl milliQ) and incubating the restriction-ligation mix at 37°C for another 3 hours. Next, the restriction-ligation mix was heat inactivated (10 min at 65°C) and two-fold diluted. Polymerase chain reactions (PCR) amplifications were performed on a T-gradient thermocycler (Biometra) in two separate amplification steps. Firstly, pre-amplification reactions were conducted in a 25µl volume (containing 2.5µl 2-fold diluted restriction-ligation mix, 0.75µl *Eco*RI/+A-primer (50ng/µl; Biolegio), 0.75µl *Mse*I/+C-primer (50ng/µl; Biolegio), 1µl dNTP's (5mM), 1.25µl $MgCl_2$ (50mM; Bioline), 2.5µl 10x reaction-buffer (Bioline), 0.5µl Taq-polymerase (1U/µl; Bioline) and 15.75µl milliQ), with the following temperature profile: an initial denaturation step of 2 min 94°C; followed by 29 cycles of 30s at 94°C (denaturation), 60s at 56°C (annealing), 60s (+1s/cycle) at 72°C (elongation); concluded by 2 min 72°C. Selective amplifications were performed in a 20 µl volume (containing 5µl 50-fold diluted pre-amplification mix, 1µl *Eco*RI/+*n*-primer (6ng/µl; IR-dye labelled; Biolegio), 0.6µl *Mse*I/+*n*-primer (50ng/µl; Biolegio), 0.8µl dNTP's (5mM), 1µl $MgCl_2$ (50mM; Bioline), 2µl 10x reaction-buffer (Bioline), 0.4µl Taq-polymerase (1U/µl; Bioline) and 9.2µl milliQ), with the following temperature profile: an initial denaturation step of 2 min 94°C; 13 cycles of 30s at 94°C, 60s at 65°C (-0.7°C/cycle), 60s at 72°C; 25 cycles of 30s at 94°C, 60s at 56°C, 60s at 72°C; concluded by 2 min at 72°C. Selective amplification reactions were performed with two primer combinations, *Eco*RI-ACC/*Mse*I-GCG and *Eco*RI-AC/*Mse*I-GCA (with IR-700 or 800 dye-labelled *Eco*RI-primers; Biolegio). All PCR reactions were performed on a T3 thermocycler (Biometra®), using a ramping speed of 1°C/s. Fragment separation took place on a model 4200IR^2 DNA Analyser (LI-COR), using 25cm denaturing gels with 6.5% polyacrylamide. IRDye size standards (50-700 bp) were included for sizing of the fragments. AFLP band patterns were scored (1 as present, 0 as absent) using the SAGA™ Automated AFLP® Analysis Software (LI-COR). Thirty randomly selected samples were analysed 2 times to test the reproducibility of the AFLP protocol. Only fragments that yielded clear and reproducible bands were retained for further statistical analyses.

Genetic analyses

Intrapopulation diversity - Within each population clones were identified by searching for pairs of ramets with identical AFLP genotypes, using the program GENOTYPE (Meirmans & van Tienderen 2004). Genotypic diversity within populations was calculated as the proportion of distinguishable genotypes $P_G=G/N_r$, with G the number of identified genets and N_r the number sampled ramets (Ellstrand & Roose 1987; Widén *et al.* 1994). Within populations, recurrent genotypes were removed from all further analyses. Genetic variation within populations was assessed by calculating

Shannon's index of diversity (*I*; Shannon & Weaver 1949), the number of polymorphic loci (N_{PL}) and the percentage of polymorphic loci (P_{PL}), for both the total data set as well as for each primer pair separately, using the software program POPGENE v.1.31 (Yeh *et al.* 1997). To assess whether the location of populations along the river course affected measures of genetic variation within populations, we performed linear regression analyses of P_{PL} on the geographic distance along the river course, for each primer pair separately, using SPSS 13.0 (SPSS Inc.).

Regional population structure - Several different approaches were used to assess the regional population structure. Firstly, we tested the null hypothesis that the nine populations constitute a single panmictic unit. To this end an Analysis of Molecular Variance (AMOVA) was performed to assess the degree of molecular variation within and among populations, using the program ARLEQUIN v.2.000 (Schneider *et al.* 2000), which performs a nested ANOVA using the matrix of Euclidean genetic distances as input (Excoffier *et al.* 1992). Secondly, the level of genetic population subdivision was estimated by calculating pairwise genetic distances between populations using Φ statistics, that are directly analogous to Wright's F statistics (Excoffier *et al.* 1992). Exact tests of population differentiation were calculated with the program Tools For Population Genetic Analysis (TFPGA) v.1.3 (Miller 1999). Analyses were performed with pairwise combinations of populations (using 20 batches and 2000 permutations), based on observed marker frequencies and assuming linkage equilibrium among loci (Miller 1999). The relationship between pairwise genetic distance (Φ_{ST}) and geographic distance was assessed with a Mantel test implemented in FSTAT v.2.9.3.2 (Goudet 1995). Thirdly, the regional population structure was examined with STRUCTURE v.2.1 (Pritchard *et al.* 2000), which employs a fully Bayesian clustering procedure that does not require a priori assignment of individuals to geographical locations. STRUCTURE v.2.1 uses a MCMC approach to cluster individuals into K panmictic groups, by minimizing deviations from Hardy-Weinberg equilibrium and linkage equilibrium. The program calculates an estimate of the posterior probability of the data for a given K, $\Pr(X|K)$ (Pritchard *et al.* 2000). The program AFLP-SURV v.1.0 (Vekemans *et al.* 2002) was used to create input files for STRUCTURE v.2.1 (Pritchard *et al.* 2000). In order to quantify the amount of variation of the likelihood for each K we performed a series of 5 independent runs for each value of K, with K ranging from 1 to the number of geographical sampling locations (N_P) plus one. We assumed a no-admixture model (Pritchard & Wen 2000) with correlated allele frequencies (Falush *et al.* 2003), using a length of the burn-in and MCMC iterations of 10 000 each (Evanno *et al.* 2005). Longer burn-in and MCMC iterations did not significantly change the results. It has been shown that in many cases $\Pr(X|K)$ may still increase slightly, even after the real K is reached (Pritchard & Wen 2004; Evanno *et al.* 2005), making inferences of K solely based on the highest values of $\Pr(X|K)$ difficult. We therefore used Evanno *et al.*'s (2005) ad hoc statistic, ΔK, which is based on the second order rate of change of $\Pr(X|K)$ with respect to K ($\Delta K = m(|L(K+1)-2L(K)+L(K-1)|)/s[L(K)]$). This ad hoc statistic ΔK should show a clear peak at the uppermost hierarchical level of structure at the true value of K (see Evanno *et al.* 2005, for a detailed description).

Dispersal – Fully Bayesian assignment programs, such as STRUCTURE v.2.1, implicitly assume that all true populations of origin have been sampled (Manel *et al.* 2002, 2005). As a result, they do not take into account that some individuals may originate (as a result of recent migration)

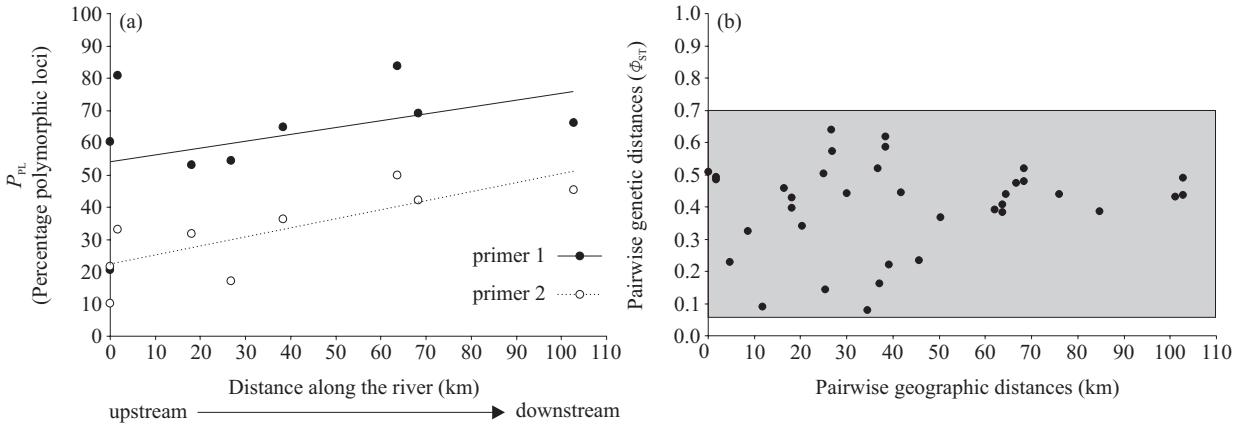


Fig 1 *(a)* The percentage of polymorphic loci (PPL) of primer-pair 1 (E-ACC/M-GCG, black circles) and primer-pair 2 (E-AC/M-GCA, white circles) in each of the nine *Sparganium emersum* populations sampled along the Niers river (Germany – the Netherlands). The lines represent the linear regression lines for primer 1 (solid line) and primer 2 (dashed line) (see text for significancies). *(b)* Correlation between pairwise genetic distances (Φ_{ST}) and geographic distances among the nine *S. emersum* populations (see text for significances).

from source locations that were not studied (*e.g.* other populations in the Niers River or even nearby catchments). To identify potential immigrants from outside our study populations, as well as migration events between the study populations, we used a frequency-based assignment procedure for dominant markers implemented in AFLPOP v.1.1 (Duchesne & Bernatchez 2002), using the 'leave-one-out' procedure. AFLPOP allocates individuals on the basis of log-likelihoods and associated *P*-values obtained from simulations. To assess the strength of the assignments, the allocation of individuals was performed in three different assignment analyses, each time using a different minimal log-likelihood difference (MLDs of 0, 1, and 2, respectively). An MLD of 0 means that a genotype is allocated to the population in which it has the highest likelihood, whereas an MLD of 2 means that a genotype has to be 10^2 times more likely to be found in population X than in any other population in order to be allocated to population X. Although a higher MLD will thus yield more reliable allocations, it will also result in a higher rate of non-assignable individuals (Duchesne & Bernatches 2002). The assignment outcome of an individual can fall into either of four groups: Firstly, correctly assigned individuals (CA), *i.e.* individuals assigned to their population of origin (the likelihood is at least 10^{MLD} times higher in their own population as in the next most likely candidate population, and the *P*-value is larger than the threshold value of 0.001). Secondly, mismatched assigned individuals (MA), *i.e.* individuals assigned to a study population other then their population of origin (likelihood more than 10^{MLD} times higher in one of the other study populations, and $P>0.001$). Thirdly, ambiguously assigned individuals (AA), *i.e.* assigned to more than one study population (the difference in likelihoods of assignment between, at least, two study populations, is smaller than 10^{MLD}). Finally, non-assignable individuals (NA), *i.e.* individuals whose likelihoods are so low that associated *P*-values fall below the threshold value of 0.001; therefore, these individuals are likely to originate from populations other than the study populations (immigrants; Duchesne & Bernatchez 2002; Berry *et al.* 2004).

Results

Genotypic and genetic variation within populations

The two AFLP primer pairs generated over 200 band fragments (in 283 individuals from 9 populations), of which a total of 156 were found to produce clear and reproducible bands and were used for further statistical analyses. Of the 283 analysed ramets, a total of 272 genets could be distinguished (Table 2): 6 genets were found two times (2 in GT, 1 in GS, 1 in HU and 2 in OE) and 1 genet was found 6 times (in OE). In this study, clones (ramets with identical AFLP band patterns) were only found within populations, not between them. Genetic diversity within populations was relatively high, with a total percentage of polymorphic loci (P_{PL}) over all populations of 83.33% (mean = 44.80%; range = 21.15-64.74) and a Shannon's diversity index (I) of 0.3338 (mean = 0.1840; range = 0.0674-0.2846). Genetic diversity within populations (measured as P_{PL}, Table 2) appeared to increase from upstream to downstream locations along the longitudinal course of the Niers River (Fig. 1a). However, this was only significant for primer-pair 2 (R^2=0.676, P=0.007) and not for primer-pair 1 (R^2=0.211, P=0.214).

Regional population structure

The AMOVA analysis showed that the overall population differentiation was high (Φ_{ST}=0.4032, P<0.0001), indicating that the populations did not form a single panmictic unit. Of the total genetic variation partitioned in the nine *S. emersum* populations, 40.32% was attributed to the differences among populations, whereas 59.68% was attributed to the differences among individuals within populations (Table 3). The pairwise genetic distances (Φ_{ST}) between populations varied widely, ranging from 0.08964 (between OE and WA) to 0.58567 (between GT and WA; Table 3). Exact tests of pairwise population differentiation suggested that nearly all of the population pairs were significantly differentiated (at the P<0.001 level), except for SW-GE (P<0.05) and OE-WA (P>0.05; Table 4). There was no clear association between pairwise genetic distances (Φ_{ST}) and geographic distances (Mantel test: R^2=0.0205; P=0.4240). At river distances < 50 km the pairwise genetic distances (Φ_{ST}) were highly variable, but at distances > 50 km the pairwise Φ_{ST} values were all relatively high (Fig. 1b). The STRUCTURE v.2.1 analyses revealed a clear peak in Evanno *et al.*'s (2005) ad hoc statistic ΔK at K = 5, corresponding to a mean (±SD over 5 runs) Pr($X|K$) of -9554.2 (±57.9). This suggests that the nine populations in the Niers River comprise of 5 clusters of populations, with the populations in each cluster more or less acting as a single genetic unit (Table 5; Fig. 2): C1 (GT and GS), C2 (KH), C3 (HU), C4 (OE, WA and KE) and C5 (SW and GE).

Dispersal

The three frequency-based assignment tests performed in AFLPOP v1.1 (Duchesne & Bernatchez 2002) showed that as the MLD increased, the number of correctly assigned (CA) and mismatched

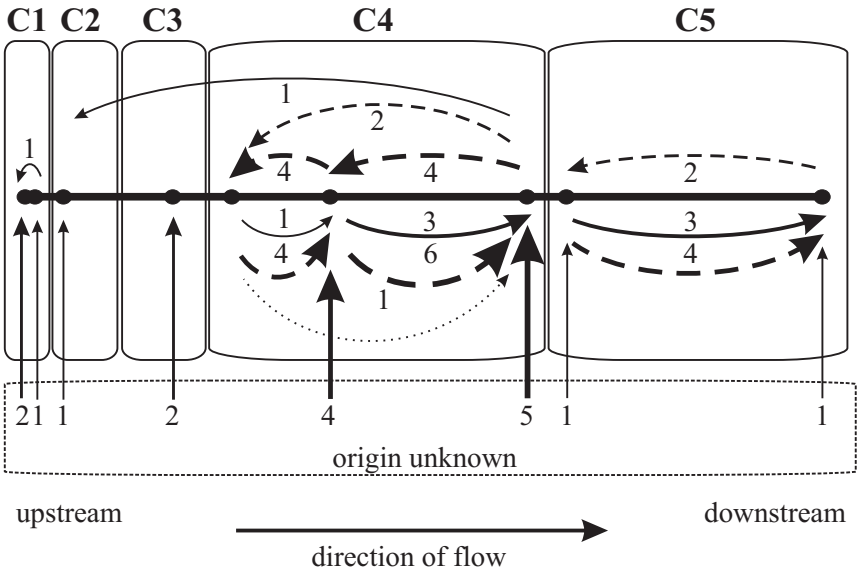


Fig 2 Schematic representation of the population structure and dispersal among the nine subpopulations of *Sparganium emersum* in the Niers River (Germany – the Netherlands). The thick black line represents the Niers River, and the black dots indicate the location of the nine populations. The five solid-lined boxes represent the five clusters (C1-C5) inferred from the STRUCTURE analyses. The arrows between subpopulations indicate dispersal events inferred from the AFLPOP analyses: arrows to the left indicate dispersal events in an upstream direction, arrows to the right dispersal in a downstream direction. Solid arrows represent unambiguous dispersal events (MA individuals, using an MLD of 2). Dashed arrows represent ambiguous dispersal events (MA individuals using an MLD of 0, but AA individuals using and MLD of 2). The vertical arrows represent dispersal events originating from unknown (non-studied) populations.

assigned (MA) individuals decreased while the number of ambiguously assigned (AA) individuals increased (the number of non-assignable individuals remaining unaffected), particularly for populations OE, WA, KE, SW and GE (Table 6). The advantage of performing three assignment tests using three different MLDs, is that it allowed inferences of, not only the unambiguous assignments, but also of the ambiguous assignments and hence about population structure. Since, population pairs with many ambiguous assignments are likely to be little differentiated, the results of the frequency-based assignment in AFLPOP v.1.1 concur with the number of K=5 population clusters inferred from the fully Bayesian-based assignment analyses in STRUCTURE v.2.1. Although, populations KH and HU are more isolated, population clusters C1(GT-GS), C4(OE-WA-KE) and C5(SW-GE) have considerable (ambiguous and unambiguous) dispersal among the populations within clusters. Moreover, there is less extensive dispersal between the 5 clusters (Table 6).

The assignment tests show that 65.4 to 80.5% of the individuals were assigned to their population of origin (using an MLD of 0 and 2, respectively), suggesting local recruitment (see numbers on the diagonal in Table 6). The assignment tests further showed that 8.1% to 2.6% of the individuals were assigned to an upstream located population (see below the diagonal), suggesting that these individuals result from dispersal in a downstream direction. Likewise, the assignment tests show that 5.5% to 0.7% of the individuals were assigned to a downstream located population (above the diagonal), suggesting that these individuals were the result of dispersal in an upstream direction. Finally, the assignment tests show that 6.25% of the individuals could not be assigned

Table 2 Genetic and genotypic diversity statistics for each of the 9 *Sparganium emersum* populations in the Niers river (*Nr* = number of ramets sampled in each population, *G* = number of genotypes identified, *P* = proportion of distinguishable genotypes, N_{PL} = number of polymorphic loci, P_{PL} (%) = percentage of polymorphic loci, and *I* = Shannon's diversity index).

			Genotypic Diversity			Genetic Diversity (recurrent genotypes excluded)					
						E-*ACC*/M-*GCG* (68)			E-*AC*/M-*GCA* (88)		
Population	Geographic Coordinates	Assigned Code	N_r	G	P_G	N_{PL}	P_{PL}(%)	I (±SD)	N_{PL}	P_{PL}(%)	I (±SD)
Güdderath tunnel	51°8'N, 6°26'E	GT	19	17	89.5	41	60.29	0.2323 (0.2479)	9	10.23	0.0314 (0.1079)
Güdderath sluis	51°8'N, 6°26'E	GS	12	11	91.7	14	20.59	0.0700 (0.1624)	19	21.59	0.0654 (0.1432)
Kamphausener Höhe	51°8'N, 6°27'E	KH	38	38	100	55	80.88	0.4345 (0.2592)	29	32.95	0.1687 (0.2673)
Hülsdonk	51°16'N, 6°26'E	HU	38	37	97.4	36	52.94	0.2072 (0.2537)	28	31.82	0.1230 (0.2164)
Oedt	51°19'N, 6°22'E	OE	39	32	82.1	37	54.41	0.1825 (0.2331)	15	17.05	0.0576 (0.1556)
Wachtendonk	51°25'N, 6°20'E	WA	39	39	100	44	64.71	0.2247 (0.2333)	32	36.36	0.1349 (0.2246)
Kevelaer	51°35'N, 6°15'E	KE	36	36	100	57	83.82	0.3744 (0.2520)	44	50.00	0.1843 (0.2306)
Schloß Wissen	51°37'N, 6°13'E	SW	36	36	100	47	69.12	0.3091 (0.2699)	37	42.05	0.1651 (0.2476)
Gennep	51°41'N, 5°58'E	GE	26	26	100	45	66.18	0.3154 (0.2766)	40	45.45	0.1894 (0.2490)
All Populations			283	272	96.1	66	97.06	0.4468 (0.2037)	64	72.73	0.2465 (0.2057)

Table 3 Analysis of Molecular Variance (AMOVA) for 272 individuals among and within nine *Sparganium emersum* populations in the Niers River (Germany - the Netherlands).

Source of variation	*d.f.*	Sum of squares	Variance components	% of variation	*P*
Among populations	8	1711.359	6.82845	40.32	< 0.00001
Within populations	263	2658.431	10.10810	59.68	< 0.00001
Total	271	4369.790	16.93656		

Table 4 Pairwise genetic distances (Φ_{ST}; below the diagonal) and geographical (*i.e.* river) distances (km; above the diagonal) for the 9 *S. emersum* populations along the Niers river (Germany – the Netherlands). Values given in bold indicate a significant differentiation (* and *** indicate a significance at $p<0.05$ and $p<0.001$, respectively).

	GT	GS	KH	HU	OE	WA	KE	SW	GE
GT		0.070	1.690	18.090	26.715	38.365	63.665	68.365	102.740
GS	**0.50926*****		1.620	18.020	26.645	38.295	63.595	68.295	102.670
KH	**0.48407*****	**0.49379*****		16.400	25.025	36.675	61.975	66.675	101.050
HU	**0.42987*****	**0.39784*****	**0.45886*****		8.625	20.275	45.575	50.275	84.650
OE	**0.57406*****	**0.63893*****	**0.50278*****	**0.32618*****		11.650	36.950	41.650	76.025
WA	**0.58567*****	**0.61751*****	**0.52105*****	**0.34119*****	0.08964		25.300	30.000	64.375
KE	**0.40880*****	**0.38295*****	**0.39084*****	**0.23436*****	**0.16292*****	**0.14325*****		4.700	39.075
SW	**0.51913*****	**0.48027*****	**0.47592*****	**0.36922*****	**0.44559*****	**0.44229*****	**0.22988*****		34.375
GE	**0.49155*****	**0.43747*****	**0.43207*****	**0.38614*****	**0.43920*****	**0.43871*****	**0.22145*****	**0.08115***	

Table 5 The proportion of individuals from each sample location assigned to each of the clusters (*K*) inferred from the STRUCTURE analysis. Proportions greater than 0.5 are given in bold.

	Inferred population clusters				
Population	C1	C2	C3	C4	C5
GT	**1.000**	0.000	0.000	0.000	0.000
GS	**1.000**	0.000	0.000	0.000	0.000
KH	0.000	**0.987**	0.012	0.000	0.000
HU	0.000	0.000	**0.757**	0.243	0.000
OE	0.000	0.000	0.000	**0.952**	0.048
WA	0.000	0.000	0.000	**0.956**	0.044
KE	0.000	0.000	0.000	**0.641**	0.359
SW	0.000	0.000	0.000	0.000	1.000
GE	0.000	0.000	0.000	0.000	**1.000**

to any of the study populations, and should therefore be considered to be immigrants from an unknown origin (either from non sampled populations within the Niers River catchment, or from other lakes or catchments nearby).

Discussion

Rather than nine separate independent populations, the Bayesian-based clustering procedure revealed an uppermost hierarchical population structure of K=5 populations. This clustering was supported by the genetic distances (Φ_{ST}) between populations, which were considerably lower among populations within clusters compared to distances among populations from different clusters. The clustering into 5 clusters was also strongly supported by the frequency-based assignment tests, which showed many mismatched assigned and ambiguously assigned individuals within and very few between clusters (Table 6). Moreover, since the number of ambiguously assigned individuals is negatively related to the genetic differentiation between populations, the larger number of ambiguously assigned individuals among populations within clusters is indicative of a lower genetic differentiation among these populations (concurring with the lower Φ_{ST} values; Waser & Strobeck 1998; McDonald 2003).

Assignment tests have rapidly become popular statistical tools for inferring dispersal on ecological time-scales (Manel *et al.* 2002, Berry *et al.* 2004; Paetkau *et al.* 2004; Manel 2005). A major advantage of assignment tests is that they yield estimates of the frequency, as well as the direction, of dispersal among populations. In linear ecosystems, such as rivers, seeds and vegetative fragments may be transported in three directions: downstream, upstream and between catchments. In the present study, the assignment tests suggested that 65.4 - 80.5 % of the individuals resulted from local recruitment in their own populations (the seeds may have sunk before they could be dispersed); 2.6 – 8.1 % was due to dispersal in a downstream direction, *e.g.* by means of water currents (hydrochory) or animals (zoochory)); and 0.7- 5.5 % was attributed to dispersal in an upstream direction. Pollux *et al.* (2005, 2006) have shown that seeds of *S. emersum* can survive a passage through the digestive tract of both fish and waterfowl, and they suggested that these animals may promote dispersal (via endozoochory) to upstream located populations. In addition, 6.25% could not be assigned to any of the study populations, and hence, most likely resulted from dispersal coming from non sampled populations in the Niers River catchment or even from populations inhabiting nearby lakes or catchments (by overland animal-mediated dispersal).

The observed dispersal events were probably not pollen-mediated because, typically, pollen-mediated gene flow seems to be effective only within several tens of meters, declining exponentially with increasing distance (Richards *et al.* 1999; Tero *et al.* 2003). Moreover, pollen dispersal principally occurs in a radial fashion (in all directions away from the parent plant), and hence, in a river system, should lead to equal dispersal in upstream and downstream directions. However, in this study the results indicate an asymmetry in the dispersal pattern: firstly, assignment tests revealed an almost four-fold higher dispersal rate in a downstream direction, compared to the dispersal in an upstream direction. Secondly, in riparian plant species, with hydrochory as their main dispersal

Table 6 Assignment of 272 *S. emersum* individuals from 9 locations in the Niers River, using 3 different minimum log-likelihood differences for the allocation of individuals (MLD of 0, 1 and 2, respectively). Given are the total number of individuals (*i.e.* genets) collected in each population, the number of correctly assigned (CA; P>0.001, and likelihood of assignment is highest to the population of origin), the number of mismatched assigned (MA; P>0.001, and likelihood of assignment is highest in a population other than the population of origin), the number of ambiguously assigned (AA; P>0.001, but the likelihood of assignment to the most likely and the second most likely population is smaller than 10^{MLD}), and the number of not-assignable (NA; P<0.001) individuals.

From \ To	GT	GS	KH	HU	OE	WA	KE	SW	GE	Total # ind	CA (#)	MA (#)	AA (#)	NA (#)
GT	14, 14, 14	1, 1, 1	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	17	14, 14, 14	1, 1, 1	0, 0, 0	2
GS	0, 0, 0	10, 10, 10	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	11	10, 10, 10	0, 0, 0	0, 0, 0	1
KH	0, 0, 0	0, 0, 0	36, 36, 36	0, 0, 0	0, 0, 0	0, 0, 0	1, 1, 1	0, 0, 0	0, 0, 0	38	36, 36, 36	1, 1, 1	0, 0, 0	1
HU	0, 0, 0	0, 0, 0	0, 0, 0	35, 35, 35	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	37	35, 35, 35	0, 0, 0	0, 0, 0	2
OE	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	26, 22, 16	4, 0, 0	2, 1, 0	0, 0, 0	0, 0, 0	32	26, 22, 16	6, 1, 0	0, 9, 16	0
WA	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	5, 3, 1	26, 23, 6	4, 1, 0	0, 0, 0	0, 0, 0	39	26, 23, 6	9, 4, 1	0, 8, 28	4
KE	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	1, 1, 0	9, 7, 3	21, 19, 19	0, 0, 0	0, 0, 0	36	21, 19, 19	10, 8, 3	0, 4, 9	5
SW	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	33, 31, 28	2, 1, 0	36	33, 31, 28	2, 1, 0	0, 3, 7	1
GE	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	7, 6, 3	18, 15, 14	26	18, 15, 14	7, 6, 3	0, 4, 8	1

strategy, asymmetric unidirectional gene flow may lead to erosion of genetic diversity in upstream river stretches and accumulation of genetic diversity in downstream stretches (Barrett *et al.* 1993). In this study, regression analyses of genetic diversity (P_{PL}) within *S. emersum* populations against the longitudinal course of the Niers River revealed a significant increase of genetic diversity towards downstream located populations, strongly suggesting an asymmetry in the rate of dispersal with dispersal occurring predominantly in a downstream direction (Gornall *et al.* 1998; Lundqvist & Andersson 2001; Liu *et al.* 2006).

S. emersum is also capable of vegetative spread (Boedeltje *et al.* 2003, 2004) and studies have shown that floating leaf fragments remain viable for up to 10 weeks (Barrat-Segretain *et al.* 1998; Barrat-Segretain *et al.* 1999), offering a potentially important mechanism of plant dispersal (Riis & Sand-Jensen 2006). However, of the 283 sampled shoots, not a single pair of ramets with identical genotypes (clones) was found in two or more spatially separated populations, suggesting that in the Niers River the dispersal and subsequent establishment of vegetative propagules may not frequently occur.

In conclusion, the very pronounced genetic differentiation among patches strongly argues against the existence of a single panmictic *spatially extended population* (Table 1), while the inference of a considerable amount of migration between the studied patches, as well as the identification of a number of immigrants originating from populations that were not sampled, argues against the presence of a *regional ensemble* (Table 1). Thus, the genetic analyses strongly suggest that the population structure and dispersal patterns (graphically depicted in Fig. 2) are in agreement with Freckleton & Watkinson's *metapopulation* model: a clear genetic population differentiation, highly variable Φ_{ST} values and dispersal between the populations (Fig. 2). Although the results did not reveal a significant pattern of isolation by distance, they did show that at distances exceeding 50 km all pairwise populations were invariably highly differentiated, suggesting that at these distances gene flow might become more constrained by distance.

General Conclusions

The results of this study show that genetic analyses may be helpful in assessing the regional population structure. The formulation of a number of testable predictions, about the genetic structure of plant populations and the rate of gene flow between them, may help to distinguish between the three main models of regional population structure proposed by Freckleton & Watkinson (2002). However, the observed distribution of genetic variation, both within and among subpopulations, only allow inferences about the relative importance of the homogenizing (gene flow) and differentiating (drift, mutation, natural selection) micro-evolutionary forces that have acted throughout *the history* of the subpopulations (Slatkin 1985; Tero *et al.* 2003). This statement may seem trivial for populations at equilibrium in a stable and predictable environment, but may be particularly important in populations that have recently been subjected to severe, unpredictable (anthropogenically induced) disturbances. Therefore, the results of population genetic analyses should be interpreted carefully, preferably in combination with information about the history of the populations and the ecology of the study species.

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Chapter 8

General Discussion

Dispersal plays a fundamental role in the life-history of plants, affecting their biology and ecology, and largely determines the regional (genetic) structure and dynamics of plant populations. However, river systems offer special environments for plants and their dispersal because of the continuous subjection of above ground plant parts to turbulent flow, the one-dimensional linear arrangement of populations along the longitudinal course of a river, and the unidirectional nature of the water flow. This General Discussion provides a synthesis of the key processes involved in the dispersal of aquatic plants inhabiting river systems. This chapter aims at addressing the questions posed in the general introduction and concludes with some suggestions for future research.

Environmental conditions, mode of reproduction and genotypic diversity

Variability in environmental conditions may lead to phenotypic variation among plant populations (Clausen *et al.* 1948), potentially affecting their ability for sexual reproduction. Across environments, these differences in sexual versus asexual reproduction may translate into genotypic variation among populations.

Plant morphology of *S. emersum* is related to water depth and current velocity, as shown by a survey in the Swalm and Rur rivers (Fig 1a; Pollux unpublished data). Here, we observed a gradual shift in plant morphology with increasing depth and current velocity from (i) the emerse type, which has emergent leaves and often a seed-bearing stem, to (ii) the floating type, which has leaves that reach, and float on, the surface but do not protrude from the water, and finally to (iii) the submerse type, which has submerged leaves only (Fig 1a). Moreover, we showed that *S. emersum* displayed a few additional morphological adaptations to increased water velocity in order to minimize the hydraulic stress (see also Haslam 1978; Chambers *et al.* 1991; Sand-Jensen 1998; Schutten & Davy 2000; Boeger & Poulson 2003; Puijalon & Bornette 2004; Asaeda *et al.* 2005; Puijalon *et al.* 2005): a reduction in plant biomass, an increased plant density (most likely due to a decrease in spacer length) leading to a more compact growth form which minimizes the drag stress on individual plants, and an increase in the leaf and stem flexibility which reduces the plant's frontal area.

Interestingly, these morphological adaptations have consequences for the plant's mode of reproduction. Most aquatic plants rely on wind or insect-mediated pollination and therefore have to produce emerging structures which allow plants to flower on or above the water surface in order to reproduce sexually. Consequently, only plants of the emerse type (Fig 1a) will be able to reproduce sexually. For *S. emersum* this type seems to be restricted to shallow (< 50 cm) and slow flowing (< 0.1 m s^{-1}) habitats (Fig 1a). In deeper water, or in areas with higher flow velocities, *S. emersum* may still be found but is more likely to reproduce clonally rather than sexually (Bartley & Spence 1987; Barrett *et al.* 1993; Grace 1993; Honnay & Bossuyt 2005). This concurs with our findings in Chapter 6 where we found a large variation in sexual reproduction among *S. emersum* populations in the Swalm and Rur rivers, strongly related to variation in the mean local water velocity within populations; *i.e.* sexual reproduction (as evident from local seed production) in populations that inhabited slow-flowing areas (in the Rur River), and no sexual reproduction (though possibly clonal reproduction) in populations inhabiting areas with faster running water (in the Swalm). We further

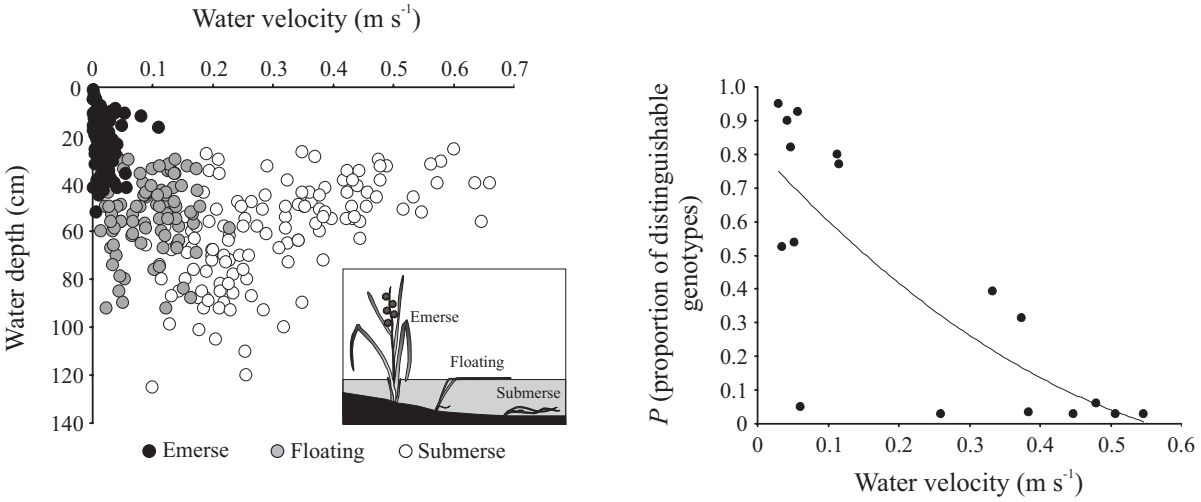

Fig 1 (a) Gradual shift in morphological types of *S. emersum* in relation to water depth and water velocity (measured 5 cm below the surface) (based on *N* = 297 plants from the Rur and Swalm rivers, during September 2005). (b) Relation between water velocity and genotypic diversity (*P*, proportion of distinguishable genotypes) within populations.

showed that sexually reproducing populations had a high genotypic diversity, while populations without local seed production either had a very low genotypic diversity or were entirely monoclonal. Interestingly, when plotting the genotypic diversity within the 17 *S. emersum* populations (*P*, the proportion of distinguishable genotypes) against the mean water velocities measured within these populations, we find a negative relationship; *i.e.* the genotypic diversity within populations tends to decrease when populations are subjected to higher water velocities, reflecting a shift in the balance between sexual versus asexual reproduction with increasing water velocity (Fig 1b).

Hydrochory: the dispersal of seeds and vegetative plant fragments by water

The importance of hydrochory for plant dispersal in rivers has mainly been studied using an empirical approach; either directly by using seed traps in order to study the dispersal of seeds, seed mimics of vegetative propagules in the field (Nilsson & Grelsson 1989; Skoglund 1990; Nilsson *et al.* 1991; Thebaud & Debussche 1991; Johansson & Nilsson 1993; Craddock & Huenneke 1997; Cellot *et al.* 1998; Andersson & Nilsson 2002; Boedeltje *et al.* 2003; Goodson *et al.* 2003; Boedeltje *et al.* 2004) or indirectly by studying the vegetation patterns along rivers and, based on these vegetation patterns, making inferences about the dispersal of plants (Nilsson *et al.* 1994; Hart & Cox 1995; Nilsson & Jansson 1995; Johansson *et al.* 1996; Danvind & Nilsson 1997; Bornette *et al.* 1998; Andersson *et al.* 2000a,b; Jansson *et al.* 2000a,b; Nilsson *et al.* 2002; Demars & Harper 2005; Jansson *et al.* 2005). In contrast, in this thesis we used both a mechanistic and a molecular approach to assess the ability for hydrochorous dispersal of *S. emersum*.

Seed floating experiments showed a surprising dichotomy in the floating capacity of *S. emersum* seeds: most seeds (~70%) sank within 3-4 weeks, while the remaining seeds (~30 %) stayed afloat for 6 months (Fig 2a; Pollux unpublished data). Germination experiments, executed at the end of

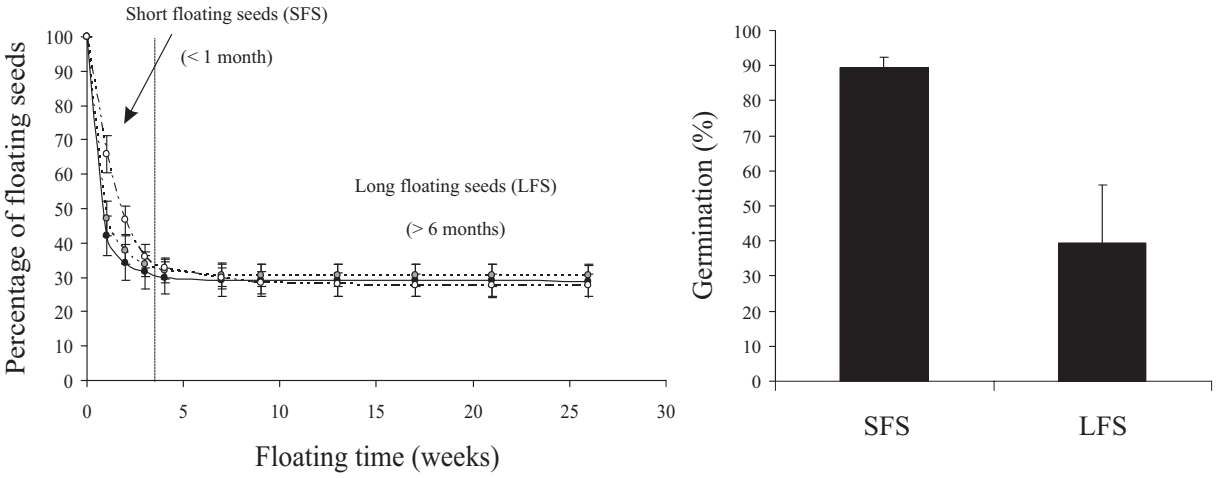

Fig 2 Preliminary results of the seed floating-experiment (Pollux *et al.* unpublished). Left. The buoyancy of *S. emersum* seeds over a period of 26 weeks. Each line represents the mean (±SE) percentage of floating seeds within an *S. emersum* population in the Rur River (*N* = 25 plants per population, setting a 100 seeds per plant to float) under dark and cold (5°C) conditions. Right. The mean (±SE) germination (%) of short floating seeds (SFS; < 4 weeks) and long floating seeds (LFS; > 6 months) (*N* = 3 populations).

the buoyancy experiment, subsequently revealed that a large proportion of both the short floating and the long floating seeds was still viable after floating for 6 months, although the long floating seeds had a significantly lower probability of germination compared to the short floating seeds (Fig 2b; Pollux unpublished data). Interestingly, the majority of the short floating seeds may not be dispersed at all, because most of the seeds that fall from the parent plants will be retained (trapped) within the dense vegetation of the population itself (Pollux personal observation). And since it may take several weeks before all the above ground plant parts have decayed and disappeared during late fall, most (if not all) short floating seeds will sink in their own population before they can be dispersed. This concurs with our findings in Chapter 7, where we found that a large proportion of individuals was assigned to their own population of origin, suggesting local recruitment (*i.e.* no dispersal). In contrast, when the dense above ground plant parts have disappeared, the long floating seeds will be washed away by the water currents and be dispersed to downstream locations, potentially over very long distances.

Apart from seeds, several studies have suggested that vegetative plant fragments of *S. emersum* can also float, and be dispersed, for extended periods (of up to 10 weeks) while remaining viable (Barrat-Segretain & Amoros 1996; Barrat-Segretain *et al.* 1998, 1999; Barrat-Segretain & Bornette 2000). Thus, the floating experiment (Pollux unpublished data) and the literature suggest that both types of propagules can be dispersed by water over long distances and become successfully established in new locations. Nevertheless, the genetic studies (Chapters 6 and 7) suggest that for *S. emersum* seed dispersal is much more important compared to the dispersal of vegetative plant fragments, which may be due to two reasons: Firstly, there may be a difference in the frequency of occurrence of seeds compared to leaf fragments in the rivers. Boedeltje *et al.* (2004), indeed showed that, in the Twentekanaal (the Netherlands), seeds of *S. emersum* were caught much more frequently than its leaf fragments. Secondly, there may be a difference in the longevity between

seeds and leaf fragments. Floating leaf fragments of *S. emersum* are highly susceptible to decay, tending to turn brown and die off in very warm (during summer) or cold water (during winter) or when becoming entangled in very shady areas, whereas seeds of *S. emersum* can float for at least 6 months while retaining their ability to germinate.

Zoochory: the dispersal of seeds by fish and waterfowl

Although water is probably the most important dispersal agent for most aquatic plants, it has been stressed that animal-mediated dispersal may also play an important role in their dispersal (Cook 1988; Barrat-Segretain 1996). Seed dispersal by animals can occur either by external adherence to feet, feathers or fur (ectozoochory; Sorensen 1986; Smith & Stiles 1994) or by ingestion of seeds (endozoochory). The probability of endozoochorous dispersal critically depends on three prerequisites: (1) the animals have to ingest the seeds in the field, (2) the seeds have to survive the passage through the intestinal tract of the animals and (3) the animals have to display migratory movements away from the parent plants.

1. Do fish and waterfowl ingest seeds?

The first prerequisite for animal-mediated seed dispersal is that the animals have to ingest the seeds of aquatic plants. There is substantial evidence from both field studies and stomach-content analyses that fish and waterbirds ingest seeds of aquatic macrophytes (Ridley 1930; Van der Pijl 1982). Stomach-content analyses have shown that many temperate European and North American fishes have seeds in their stomachs, with seed quantities in the stomachs of individual fish ranging from a few to more than a 1000 seeds per stomach (Ridley 1930; Crivelli 1981; Van der Pijl 1982; Bergers 1991; García-Berthou 2001; Nurminen *et al.* 2003; Chick *et al.* 2003; van Riel unpublished), while both field observations and stomach-content analyses have shown that many waterbirds feed on, and carry macrophyte seeds in their stomachs (*e.g.* Guppy 1906; McAtee 1918; Metcalf 1931; Martin & Uhler 1939; Anderson 1959). Among the fishes the Cyprinidae may be the most likely fish species to ingest seeds (Bergers 1991; García-Berthou 2001; Nurminen *et al.* 2003), while among the waterbirds the Anatidae (*i.e.* swans, geese and ducks), but also the coot (Fulica atra; a non-Anatidae), are the most likely bird species to feed on the seeds of aquatic macrophytes (Clausen *et al.* 2002; Figuerola & Green 2002; Green *et al.* 2002).

Many waterbirds actively search for, and forage on, macrophyte seeds (*i.e.* the obligate and opportunistic granivores), either directly by taking them from the plants or indirectly by filter-feeding while sifting through the water layer or bottom substrates (Clausen *et al.* 2002; Figuerola & Green 2002; Green *et al.* 2002). These include the two waterfowl species, teal (*Anas crecca*) and mallard (*Anas platyrhynchos*), used in Chapter 4. However, some waterbirds and probably most fish species (including carp *Cyprinus carpio*, used in Chapters 2 and 3) are non-granivores. These species may take up seeds unintentionally (*i.e.* passively) while foraging on vegetative plant parts (*i.e.* the herbivores and omnivores) or while sifting through the detritus layers on the bottom looking for

invertebrate prey (*i.e.* the zoobenthivores and omnivores) (Stiles 2000; Figuerola *et al.* 2002; Clausen *et al.* 2002; Nurminen *et al.* 2003). In fact, Figuerola *et al.* (2002) suggested that the non-intentional ingestion of seeds by herbivores, zoobenthivores and omnivores may actually be an important mode of zoochorous dispersal for temperate aquatic plants, as most aquatic plants produce non-fleshy fruits with a small proportion of edible material (*e.g. Zannichellia*, *Ruppia*, *Potamogeton*, *Sparganium*, *Sagittaria*, *Scirpus*, etc), and are therefore less likely to be actively preyed upon.

The production of non-fleshy fruits may also have important implications for the duration of the dispersal period of aquatic macrophytes (Figuerola *et al.* 2002). In terrestrial systems, the dispersal period of fleshy seeds is concentrated around a short fruiting season; as soon as the fruits have fallen on the floor and the fleshy pulp has decayed and gone, the seeds are not attractive anymore and will not be ingested and dispersed by animals. However, in aquatic systems, the period of zoochorous dispersal may extend from fall to early spring. Here, the (predominantly non-fleshy) seeds of most aquatic plants are released in fall and then either float on the water surface or sink to the bottom where they will stay available to fish and waterfowl species (regardless of whether they actively forage on the seeds or unintentionally ingest the seeds while foraging on the plant parts or on the bottom) until the germination of seeds in spring (Figuerola *et al.* 2002).

2. Do the seeds survive the intestinal tract?

The second prerequisite is that the seeds have to survive a passage through the intestinal tract of animals. The survival of seeds can be tested either by means of feeding experiments (Charalambidou & Santamaría 2002) or by collecting droppings of animals in the field and checking these for the presence of viable seeds (Figuerola *et al.* 2002; Green *et al.* 2002). Both types of study show that seeds of aquatic plant can survive the digestive tract of animals. However, when studying the factors that play a role in the survival of seeds, feeding experiments are more suitable because here you can control both the number and the type of seeds that are fed to the animals, and the ingestion of these seeds can subsequently be compared to their survival.

We showed that with increasing seed size (within plant species), the probability of ingestion decreased and the probability of seed survival during gut passage increased, while the seed retention time, the probability of germination and (arguably) the germination rate were not affected. Surprisingly, as the decrease in ingestion was counterbalanced by an equal increase in seed survival, this resulted in an overall probability of fish-mediated dispersal which was equal for all studied seed sizes of *S. emersum*. We further showed that the hard seeds of *S. emersum* had a lower probability of ingestion compared to the relatively soft seeds of *S. sagittifolia*, while the probability of survival was higher and the seed retention time tended to be longer for *S. emersum* (though this latter depended on the animal species, see below). Furthermore, the gut passage had a contrasting effect on the probability of germination and the germination rate between these two plant species: a positive effect on the probability of germination and the germination rate for the harder seeded *S. emersum*, and a negative effect for the softer seeded *S. sagittifolia*. Overall, *S. emersum* had a higher probability of dispersal, a higher potential for dispersal over longer distances, and a

higher probability of post-dispersal establishment, compared to *S. sagittifolia*. We also compared the potential for seed dispersal between different animal species. Although, the morphology of the digestive system and the physical and chemical environment in the digestive tract are known to influence the seed retention time, seed survival and seed viability (Charalambidou & Santamaría 2002), we found little differences between teal and mallard; most likely because these concern two closely related *Anas* spp. with similar digestive physiologies (Miller 1984; Charalambidou *et al.* 2003). We did, however, find considerable differences between fish (Chapter 3) and ducks (Chapter 4): The survival rate was higher for seeds ingested by fish compared to ducks, both for *S. emersum* (22.65 % for seeds ingested by ducks versus 38.58 % for seeds ingested by fish) and particularly for *S. sagittifolia* (1.60 % for seeds ingested by ducks versus 20.97 % for seeds ingested by fish), most likely because the more specialized gut of ducks provided a more hostile environment for seeds compared to the relatively unspecialized gut of fish. Moreover, the retention time of seeds was higher in ducks compared to fish, most likely because the intestinal tract of ducks is longer and has specialized features (particularly the crop and gizzard) which may retain seeds for longer periods of time (Charalambidou & Santamarí 2002). Furthermore, there were only slight differences in seed germination between seeds ingested by fish and ducks, both for *S. emersum* (79.04 % for seeds ingested by ducks versus 82.27 % for seeds ingested by fish) and *S. sagittifolia* (18.83 % by ducks versus 25.04 % by fish). This latter observation is in agreement with a review by Traveset (1998), which suggests that interspecific differences among animals may have limited effects on seed germination (see also Santamaría *et al.* 2002). Interestingly, many studies that compared the seed retention time, seed survival and seed viability of seeds ingested by different animal species found no or very little differences, which is often attributed to large intraspecific variation in the digestive characteristics of individual animals (*e.g.* Santamaría *et al.* 2002). However, intraspecific variation within animal species has hardly ever been studied. Although not specifically mentioned in Chapter 3, the repeated experiments revealed considerable intraspecific variation in the probability of ingestion and retrieval between individual carp (N = 12 experiments) (Fig 3a,b). Interestingly, carp individuals who had a high probability of ingesting *S. emersum* seeds, generally also had a high probability of ingesting *S. sagittifolia* seeds (Fig 3c). Similarly, carp individuals who had a high probability of digesting the *S. emersum*, also had a high probability of digesting the *S. sagittifolia* seeds (Fig 3d; Pollux unpublished data).

3. Do the animals display migratory movements necessary to disperse the seeds?

The third prerequisite is that the animals have to move or migrate to other locations in order to be able to disperse the seeds. Carp is generally associated with standing water bodies (floodplain lakes) and is therefore often assumed to be a non-migratory species. However, several studies have shown that coarse cyprinid fishes in rivers often show a dichotomy in their migratory behaviour, with a large proportion of the population displaying site-fidelity, typically occupying a 'home-range' within a stretch of a river (the size of this home-range being species specific; Gerking 1953; Crook *et al.* 2001; Crook 2004a,b), while a smaller proportion of the population displays long-distance

migrations (Stott 1961, 1967; Stuart & Jones 2006). In lowland rivers, for example, about 80% of the carp populations occupy a home-range of several kilometres, while a smaller proportion of the population may display larger migratory movements ranging from 100 up to 1000 km (Crook 2004a,b; Stuart & Jones 2006). Mallard and teal are migratory waterbirds that can travel great distances (*e.g.* teal has been known to travel over 1200 km in less than 24 h; Figuerola & Green 2002). However, the mallard and teal that are residing in the Netherlands during autumn and winter use these rivers and lakes as a winter habitat (Van Noorden 1992; Voslamber *et al.* 1998) and tend to display local migratory movements (*e.g.* diurnal feeding migrations) within a certain home range of several kilometres (Guillemain *et al.* 2000, 2002; Mack *et al.* 2003), rather than long-distance migrations (though individual ducks might still display migratory movements over larger distances). Thus, both fish and waterfowl are likely to disperse seeds over several kilometres, and potentially over much larger distances (see also below).

A comparison between seed dispersal by different vectors: water, fish and waterfowl

The nature of a dispersal vector may have great influence on the distance and direction of seed dispersal in the field, as well as on the shape of the dispersal curve.

The most obvious difference in the dispersal of seeds of riverine plants by different dispersal vectors (water, fish and waterfowl) is the possible dispersal *direction* of seeds: seeds that are dispersal by water currents are restricted to transportation in a downstream direction only (unidirectional dispersal), while seeds that are dispersed by fish may be transported to both upstream and downstream directions (bidirectional dispersal), and seeds that are dispersed by waterfowl may be transported, not only to upstream and downstream locations, but also overland to nearby water bodies, such as lakes or river catchments (radial dispersal).

There may also be considerable differences in the potential dispersal *distances* achieved by different dispersal vectors. The floating seeds and plant fragments of *S. emersum* may travel at an average speed of 0.05 to 0.5 m s^{-1} (according to the water velocities measured in the Swalm and Rur rivers in September 2005, Chapter 6). This means that seeds can be dispersed over distances ranging from 800 to 8000 km, during the period between the seed release in autumn and seed germination in spring (Fig 4a). Vegetative plant fragments of *S. emersum* can also float for extended periods (of up to 10 weeks) while remaining viable (Barrat-Segretain & Amoros 1996; Barrat-Segretain *et al.* 1998, 1999; Barrat-Segretain & Bornette 2000) corresponding to dispersal distances ranging from 300 to 3000 km (at a water velocity of 0.05 to 0.5 m s^{-1}, respectively). The swimming speed of fish is usually expressed in units of number of body lengths per second (bl s^{-1}), and for carp the optimum swimming speed is about 1 to 2 bl s^{-1} (note that the optimum swimming speed is of great ecological importance because fish generally swim close to this optimum speed as this is energetically most favourable) (Ohlberger *et al.* 2006). For the carp used in our experiments (with a fork length of approximately 25 cm) this would lead to an estimated optimum swimming speed between 0.9 to 1.8 km h^{-1} (see also Shin *et al.* 2003). Combining the optimum swimming speeds with information about the seed retention times in the intestinal tract of carp obtained from Chapters 2 and 3, this

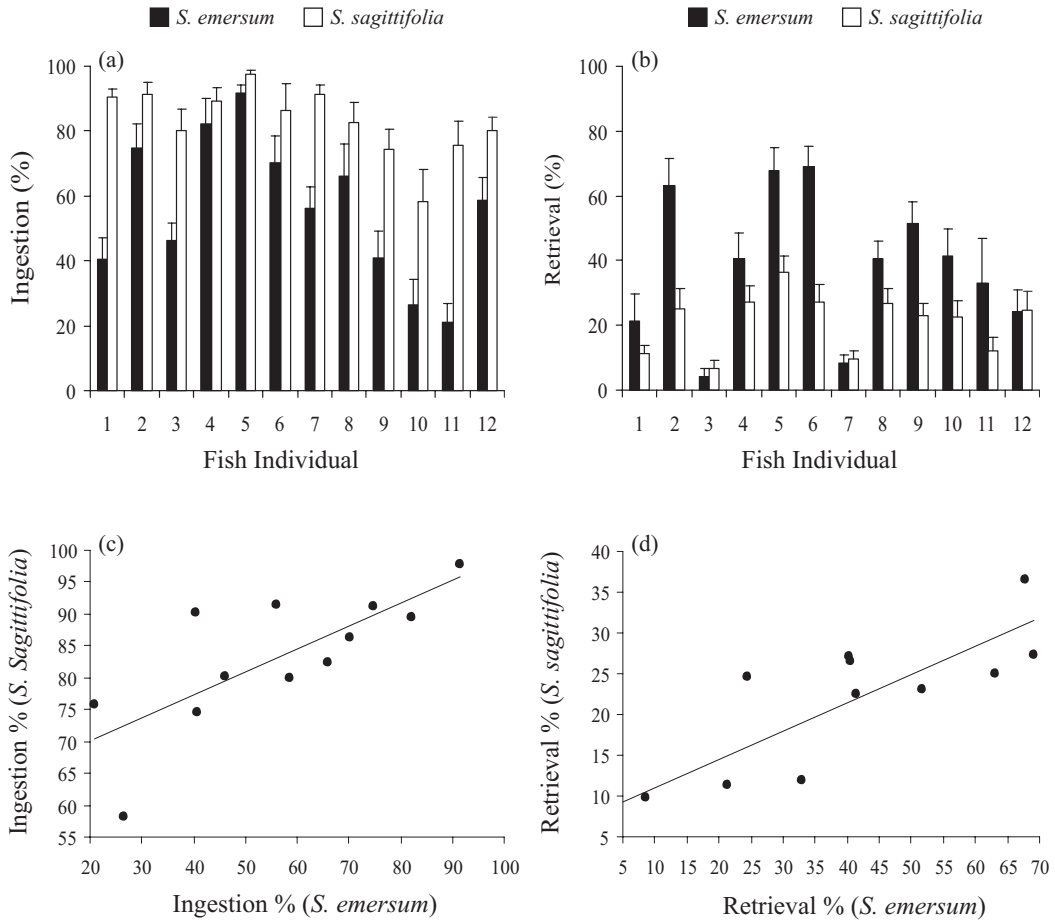

Fig 3 Intraspecific variation in the ingestion and digestion of *S. emersum* seeds among the 12 individual carp used in the experiments of Chapter 3. (a) Mean (±SE) ingestion (%), (b) mean (±SE) retrieval (%), (c) correlation between the ingestion of *S. emersum* and the ingestion of *Sagittaria sagittifolia*, and (d) correlation between the retrieval of *S. emersum* and the retrieval of *S. sagittifolia* (Pollux unpublished data).

may lead to a maximum dispersal distance of 13.5 to 27 km (Fig 4b). The flying speed for *Anas* ducks (including mallard and teal) ranges from 60 to 78 km h^{-1} (Welham 1994), which may lead to a maximum dispersal distance of 3600 to 4680 km based on the seed retention times in the intestinal tract of teal and mallard as inferred from Chapter 4 (Fig 4c; see also Charalambidou *et al.* 2003). However, it should be noted that (i) animals may not necessarily swim or fly continuously over long time periods, but instead may move over shorter distances, and (ii) the dispersal trajectories of animals may not occur in a straight line away from the source, but may follow a random, criss-cross pattern (for example when fishes are searching for food) (Charalambidou *et al.* 2003).

The *shape* of the dispersal curves also clearly differs between the three dispersal vectors. The hydrochorous dispersal curve has a very fat tail (Fig 4a), due to the presence of long floating seeds (approximately 30% of the seeds), suggesting a very high potential for long-distance dispersal. The ichthyochorous dispersal curve has a very short (to almost no) tail (Fig 4b). This is most likely due to the unspecialized gut of carp that does not allow long seed retention times in their intestinal tract (in our experiments usually less then 12 to 14 hours; Chapters 2 and 3). In contrast, the

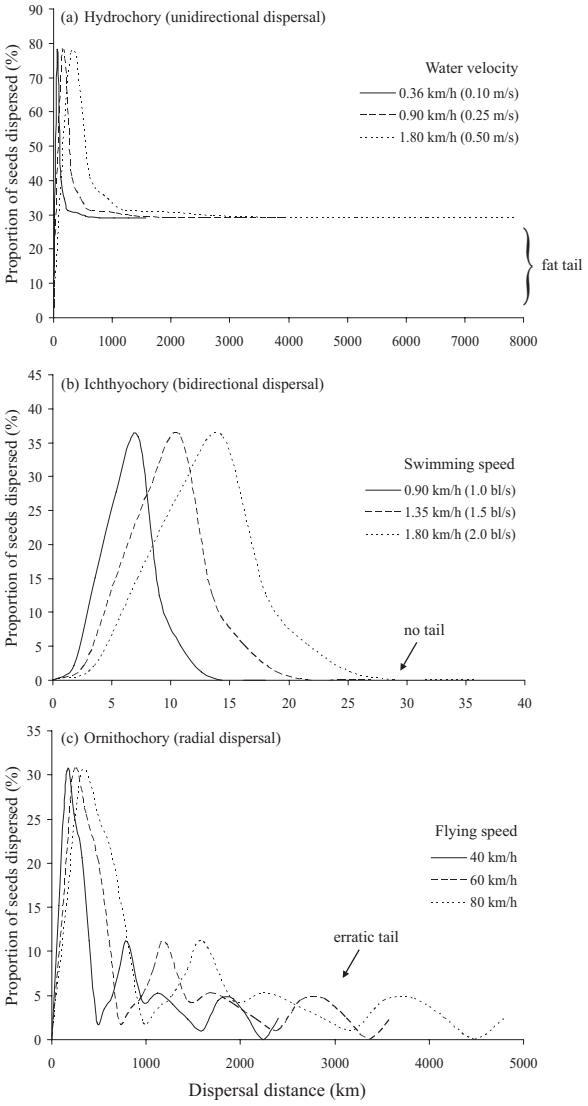

Fig 4 The dispersal curves of *S. emersum* when dispersed by different agents, based on data obtained from the experiments performed in this thesis: **(a)** Hydrochorous dispersal, based on the buoyancy of seeds (Pollux *et al.* umpublished data) and water velocities as measured in the Rur and Swalm rivers (Chapter 6). Notice the fat tail of the distribution, suggesting a high potential for long-distance dispersal. **(b)** Ichthyochorous dispersal, based on the seed retention time of *S. emersum* in the intestinal tract of carp (Chapter 2 and 3) and the optimal swimming speed of carp (see text). Notice the absence of a tail in this distribution, due to the unspecialized gut structure of carp. **(c)** Ornithochorous dispersal, based on the seed retention time of *S. emersum* in the intestinal tract of ducks (teal and mallard; Chapter 4) and the mean flying speeds of *Anas* spp. (see text). Notice the long (erratic) tail in this distribution, which is most likely due to the presence of specialized gut structures (*e.g.* the crop, gizzard). These may hold seeds for various lengths of time, resulting in an erratic (rather than a smooth) pattern of seed defecation.

ornithochorous dispersal curve does have a long, though erratic tail (Fig 4c). This is probably related to the specialized gut structures (*e.g.* crop, gizzard) which may retain seeds for various lengths of time (Charalambidou & Santamaría 2002) allowing longer seed retention times (in contrast to fish) though an erratic, rather then a smooth and continuous, pattern of seed defecation (Chapter 4).

Asymmetric dispersal and the consequences for genetic diversity within populations along the longitudinal course of rivers

Considering all of the above, one might expect to find an asymmetry in the dispersal rate between up- and downstream directions, with dispersal occurring more frequently in a downstream direction (by means of hydrochory) compared to upstream directions (by means of zoochory).

Traditionally, Wright's *F*-statistics have been used to estimate the number of migrants exchanged among populations per generation as $F_{ST} = 1/(4N_em+1)$, or equivalently $N_em = 1/4(1/F_{ST}-1)$, with N_e the effective population size of each population, m the migration rate between

populations, and hence, $N_e m$ the effective number of migrants exchanged per population (Wright 1951). The usefulness of these estimates has recently been criticized (*e.g.* Whitlock & McCauley 1999; Neigel 2002, but see Bohonak 1999), because of the many underlying, biologically unrealistic, assumptions. Of these unrealistic assumptions one is particularly important in our case, namely the inherent assumption of equal (symmetric) gene flow among populations, which is likely to be violated in unidirectional ecosystems such as rivers. Hence, F_{ST}-based estimates of gene flow will be of limited use in river ecosystems. However, there are currently two alternative approaches available that can be used to infer unequal (asymmetric) migration rates between populations (Cain *et al.* 2000): the coalescent approach (also known as the genealogical approach) and (ii) the assignment procedure. Of these two approaches, the coalescent approach implement in the software *Migrate* (Beerli & Felsenstein 1999, 2001; Beerli 2002; Beerli unpublished a,b) has several severe drawbacks: the program has a very long running time (if a large number of populations and markers are included in the data set the running time may easily take several weeks or even several months) with a realistic possibility of your computer crashing in the mean time (Beerli 2002; some authors have circumvented this problem by using only a part of their data set; Imbert & Lefevre 2003), and, more importantly, its ability to obtain reliable estimates of migration rates and associated confidence intervals has been questioned (Abdo *et al.* 2004). The assignment procedures appear to be more promising; the calculations are very rapid (taking several minutes) and the output is straightforward and easy to interpret. Consequently, assignment tests have rapidly become popular statistical tools for inferring (unequal) migration rates among populations (*e.g.* Manel *et al.* 2002; Berry *et al.* 2004; Manel 2005).

Assignment tests in the Niers River revealed an almost four-fold higher dispersal rate in a downstream direction, compared to the dispersal in an upstream direction. In the Rur River, however, gene flow could not be inferred because the nine sampled subpopulations formed a single population with little differentiation among the subpopulations. This low differentiation among subpopulations in the Rur River precludes the inference of migration, regardless of the approach that would be used (whether it would be an assignment-based approach, a coalescent approaches or an F_{ST}-based approach). Finally, in the Swalm River, gene flow could also not be assessed because the extensive clonal growth led to the formation of monoclonal populations. Here, the sampled patches were in fact single genets (each patch representing a group of genetically identical ramets) rather than populations (which would consist of genetically different shoots), precluding the inference of migration (Chapter 6).

In riparian plant species with hydrochory as their main dispersal strategy, asymmetric gene flow may affect the patterns of genetic diversity within populations along the longitudinal course of river systems. The genetic diversity within populations might be lower in upstream river stretches (due to the continuous wash down of generative propagules as well as the threat of uprooting and wash down of individuals) compared to downstream river stretches (where the continuous influx of generative and vegetative propagules may lead to an accumulation of genetic diversity; Barrett *et al.* 1993), a process which may be considered to be a genetic equivalent of the Drift Paradox (see below).

In the Niers River, regression analyses of genetic diversity within *S. emersum* populations (*i.e.*

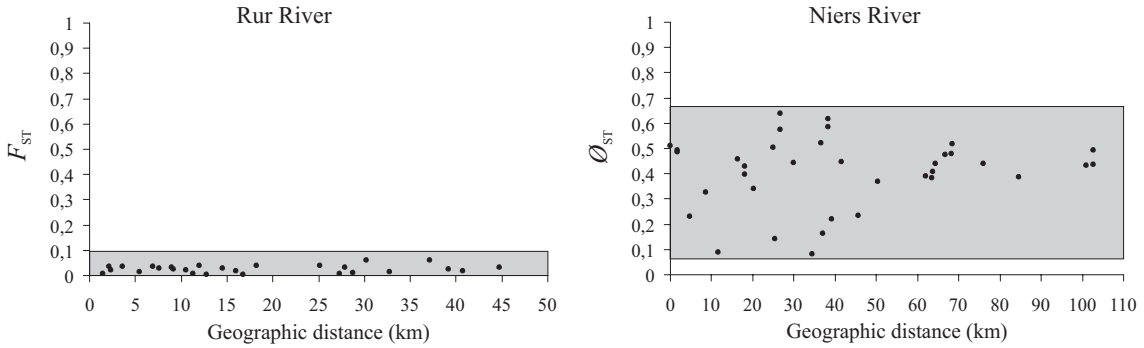

Fig 5 Correlation between pairwise genetic distances (F_{ST} and Φ_{ST}) and geographic distances among *S. emersum* populations in the Rur and Niers rivers (these correlations could not be calculated for the *S. emersum* 'populations' in the Swalm River, because most the sampled patches consisted of a single genet).

P_{PL}, percent polymorphism of AFLP markers) against the longitudinal river course did indeed reveal a significant increase of genetic diversity towards downstream located populations (Chapter 7). However, in the Rur and Swalm Rivers, associations of genetic diversity within populations (number of alleles, expected and observed heterozygosity of microsatellite markers) and the location of populations along the river course were not found (Chapter 6). In fact, most studies have failed to show a significant effect of unidirectional gene flow on the pattern of genetic variation along rivers (*e.g.* Ritland 1989; Russel *et al.* 1999; Lundqvist & Andersson 2001; Imbert & Lefèvre 2003; Tero *et al.* 2003; Prentis *et al.* 2004; DeWoody *et al.* 2004; Jacquemyn *et al.* 2006; but see Gornall *et al.* 1998; Lundqvist & Andersson 2001; Liu *et al.* 2006). This absence of increase in genetic diversity within populations, observed in most studies, may well be due to upstream (animal-mediated) dispersal of generative and vegetative propagules, which may restore genetic diversity in upstream locations by the reintroduction of alleles.

Does S. emersum act as a spatially extended population, a metapopulation or a regional ensemble? A comparison between different river systems

Plant populations can exist as a spatially extended population, metapopulation or regional ensemble (Freckleton & Watkinson 2002). Genetic analyses may help to identify the regional structure of plant populations, and to this end we formulated a number of testable hypotheses about the genetic structure and rate of gene flow for each of the three population models.

When applying this list of testable hypotheses to the *S. emersum* populations in the Rur, Niers and Swalm rivers, we found large differences in their regional structure. In the Rur River, *S. emersum* acts as a spatially extended population (Fig 6), because here we found very low and non-significant F_{ST}-values and no isolation by distance (Fig 5). This, together with the Bayesian clustering procedures, suggests the presence of a single genetically uniform population. Moreover, preliminary analyses showed that assignment tests yielded very high proportions of ambiguously assigned individuals (40-90%) making inferences of migration rates between patches impossible, as would be expected in a spatially extended population). Since in the Rur River *S. emersum* occurs in discrete patches, and

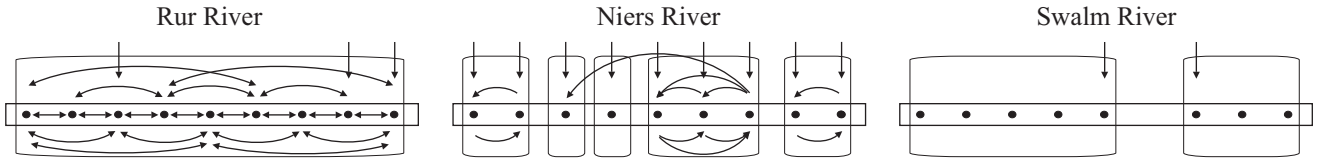

Fig 6 A schematic representation of the regional structure of *S. emersum* populations in the Rur (spatially structure local population), Niers (metapopulation) and Swalm (regional ensemble) rivers (Chapters 6 and 7).

the river most likely does not represent a uniformly extended suitable habitat, *S. emersum* likely exists as a ´spatially structured local population´, which is a type of spatially extended population (see Fig 2c of the General Introduction). In contrast, in the Niers River *S. emersum* appears to exist as a metapopulation (Fig 6). Here, we found variable Φ_{ST}-values (both high and low) and no significant isolation by distance (Fig 5). Both, the high Φ_{ST}-values and the Bayesian clustering procedures showed the presence of different populations, while the assignment tests revealed the occurrence of dispersal among populations. Finally, it is difficult to ascribe the *S. emersum* 'populations' of the Swalm River to any of the models described by Freckleton & Watkinson (2002). In fact, it is not clear whether we should speak of populations at all, because the extensive clonal growth led to the formation of monoclonal patches, which are essentially single individual plants that do not reproduce sexually. Only the two populations at the upstream and downstream edge of Lake Hariksee consist of multiple genotypes, most likely due to the introduction of seeds from nearby catchments by waterfowl. The *S. emersum* 'populations' could be divided into two clusters (separated by Lake Hariksee) and the assignment test revealed an absence of dispersal between these clusters (Fig 6). Thus, although the *S. emersum* populations in the Swalm River do not really fit into any of the models described by Freckleton & Watkinson 2002 (the aspect of clonal growth is not included in their models), they mostly resemble a regional ensemble; *i.e.* a series of highly persistent and highly isolated (no migration) populations.

The existence of very large differences in the regional structure of *S. emersum* populations that inhabit different river systems raises the question which landscape features or environmental factors cause these differences. One of the most obvious factors is water velocity, which has been shown to affect plant morphology and consequently plant reproduction. This has had a large influence on the regional structure in the Swalm River, because clonal reproduction led to the formation of predominantly monoclonal (highly persistent) patches, while the absence of sexual reproduction effectively resulted in the isolation of patches as seed-mediated exchange of migrants among populations did not take place. A second factor that might have played a role in the Swalm River is Lake Hariksee, which may have acted as a migration barrier for asexual propagules between the two regions. However, it is difficult to determine what landscape features may have resulted in the difference between the Rur and Niers rivers. This difference might be related to the presence of dams, weirs or watermills in the rivers, either in the present or in the past (Dynesius & Nilsson 1994; Nilsson *et al.* 2005), which may have negative impacts on the hydrochorous dispersal of seeds and vegetative plant fragments (Anderson *et al.* 2000a; Jansson *et al.* 2000b; but see also Jansson *et al.* 2005), as well as on the migratory movements of fish (*e.g.* Winter & Van Densen 2001; Morita

& Yamamoto 2002) and thus, undoubtedly, also on the ichthyochorous dispersal of riparian plants. Currently the Rur River is completely free of any weirs or watermills, while in the Niers River there are still a number of weirs present. However, the locations of these weirs in the Niers do not seem to correspond with the clusters that were found in the genetic analyses. It is possible that the clusters correspond to the presence of weirs or watermills in the past, however, this information is not readily available.

The Drift Paradox - the problem of plant population persistence in rivers

Aquatic organisms that inhabit river systems are continuously facing the danger of being swept downstream. Although many species have morphological or behavioural adaptations that reduce this risk (Vogel 1994), it has been suggested that in the absence of a mechanism for upstream dispersal long-term population persistence will be impossible (Speirs & Gurney 2001; Pachepsky *et al.* 2005). The 'Drift Paradox' (Müller 1954, 1982; Hershey *et al.* 1993), therefore, states that aquatic organisms must compensate for the loss of individuals due to downstream drift of seeds or larvae by upstream migration (Kopp *et al.* 2001; Humphries & Ruxton 2002). Surprisingly, however, although the unidirectional nature of river systems may be expected to have a much larger influence on sessile organisms, such as aquatic plants, which lack any means of active upstream migration, the drift paradox has never been addressed for these species.

Butcher (1933) already noted that, despite the fact that many aquatic plants are sessile the vegetation in rivers is not permanent. Natural streams and rivers are characterized by a highly dynamic hydrological regime leading to ongoing changes in the geomorphology of the riverbed (Wolfert 2001) and, consequently, in the vegetation of the riverbed (Butcher 1933; Haslam 1978; Reed *et al.* 2000; Reid & Ogden 2006). Although downstream located habitat patches that become extinct may easily be recolonized by means of hydrochoric transportation of propagules coming from upstream located populations, upstream located habitat patches that become depopulated will remain empty without a mechanism for upstream dispersal, precluding long-term plant population persistence in rivers (Fig 7; Speirs and Gurney 2001). However, the occurrence of aquatic plants in river systems all over the world poses the interesting question as to how sessile organisms are capable of maintaining persistent populations in river systems. This problem is even more pressing for non-sessile, floating plants, such as duckweeds (Lemnaceae). These plants are particularly susceptible to local 'population extinction' in rivers, due to the wash down of individuals. Nevertheless, even members of the Duckweed family are capable of colonizing and maintaining persistent populations in river systems all over the world (Uotila 1999; Hussner & Losch 2005; De Neiff & De Neiff 2006). We suggest that animal-mediated dispersal towards upstream locations may offer an explanation for the drift paradox of plant population persistence in rivers, either internally by seed ingestion or externally by adherence to feet, fur and feathers (as may be the case for members of the Duckweed family; Vasseur *et al.* 1993).

Thus, in natural heterogeneous streams with a discrete, patchy distribution of suitable habitats that are arranged linearly in space, plant population persistence may be mediated by two opposing

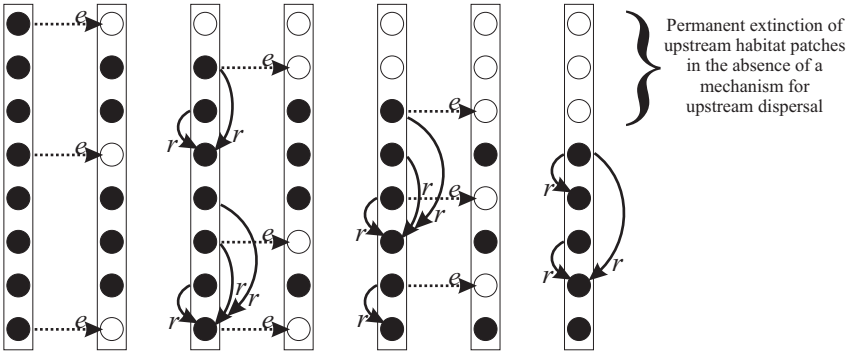

Fig 7 A schematic representation of Drift Paradox for aquatic plants in rivers, showing the permanent extinction of upstream habitat patches during a consecutive series of extinction and recolonization events, in the absence of a mechanism for upstream dispersal. The vertical rectangle represents the longitudinal course of a river, the white dots represent unoccupied suitable habitat patches, and the black dots represent occupied patches. The letter *e* denotes the occurrence of stochastic extinction events, the letter *r* the occurrence of hydrochoric re-colonisation events.

forces: on the one hand a tendency towards a downstream 'movement' of the average location of populations within the species zone (Figs 7 and 8; Speirs & Gurney 2001), and on the other hand, re-colonisation events in upstream habitat patches via zoochoric seed dispersal (Fig 8; Honnay *et al.* 2001; Higgins *et al.* 2003; Levine 2003; Purves & Dushoff 2005). Even infrequent dispersal events may be sufficient to maintain population persistence, because depopulated areas provide a competitive advantage to new recruits and hence a greater fitness and more rapid growth (Anholt 1995). This may be applicable to spatially structured local populations (Fig 2c in the General Introduction) and metapopulations (Fig 2d-f), but not to extended or patchy populations (Fig 2a-b; because here the continuous area of suitable habitat may allow populations to expand by clonal growth towards upstream locations) or to regional ensembles (Fig 2g; because here the absence of migration will preclude re-colonization of depopulated upstream locations).

Suggestions for future research

To gain more insight into the importance of different aspects of plant dispersal in rivers (directions, distances, propagules of dispersal, dispersal mechanisms), it would be helpful to study the dispersal of different plant species with clearly different dispersal characteristics: *e.g.* (i) rooted plants versus free floating species (*i.e.* the hydro- and helophytes versus the pleustohelophytes; see Table 1 of the General Introduction), (ii) plant species with very short floating times versus plant species with very long floatation times, (iii) plant species with a very high potential for zoochorous dispersal and thus also for upstream dispersal (hard seeded species) versus plant species with a very low potential for zoochorous upstream dispersal (*e.g.* Nuphar spp; Smits *et al.* 1989), (iv) plant species with water pollination (hydrophily) versus plant species with wind pollination (anemophily) and insect pollination (entomophily). Furthermore, our study shows a large variety among different river systems, and to gain more insight into the impact of various environmental conditions and landscape feature, it would be useful to study the dispersal of plants in different river systems with

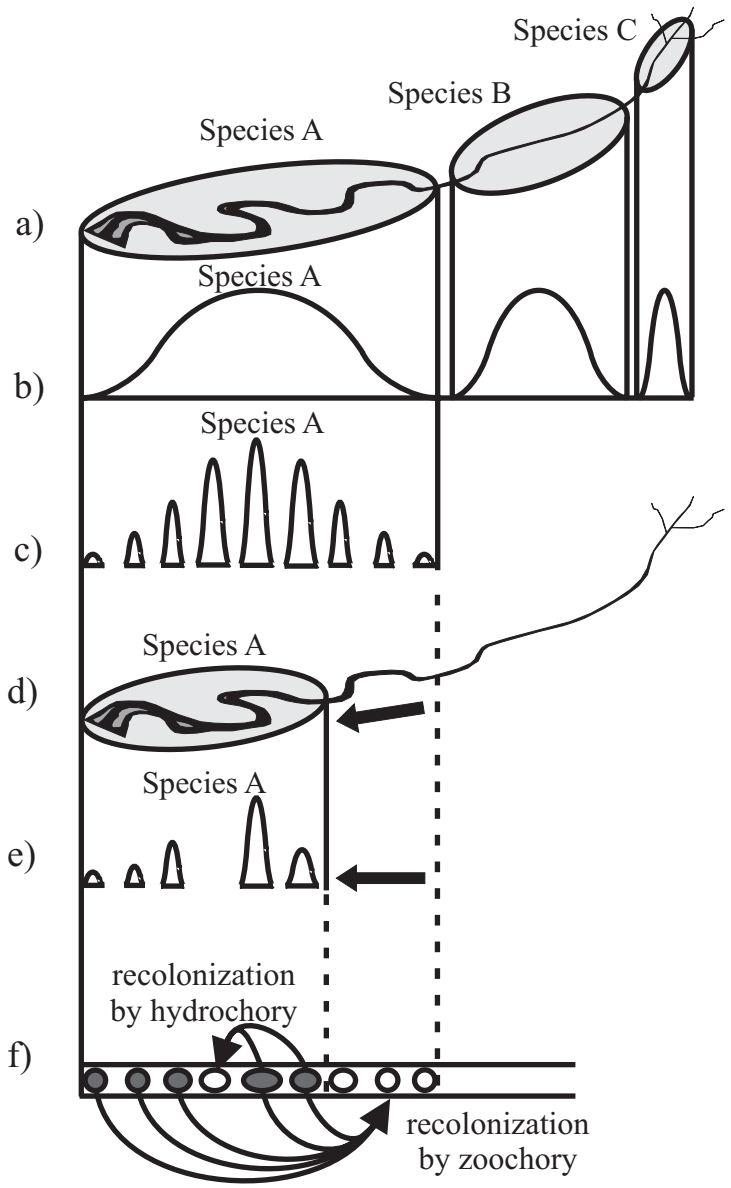

Fig 8 A conceptual representation of plant population persistence in rivers. **(a)** Zonation of species along the river course. The gray areas indicate the range of potentially suitable habitats for each species, depending on their adaptations to local environmental conditions (Hynes 1970; Haslam 1978). **(b)** Within each zone, the population abundance of a species will follow a normal distribution, along the reigning environmental gradients running from upstream to downstream along the longitudinal course of a river (*e.g.* current velocity, substrate coarseness, temperature, oxygen concentration, temperature, turbidity) (Cox and Moore 1980). **(c)** However, along natural heterogeneous rivers with a patchy distribution of suitable habitats, population abundance is more likely to follow a bell-shaped patchy pattern of normal distributions. **(d-e)** This linearly arranged 'population of populations' (sensu Levins 1969), is continuously subjected to unidirectional flow and will, in combination with unpredictable storms and floods (leading to stochastic extinctions), inevitably result in a tendency towards a downstream 'movement' of the average location of populations within the species zone (Haslam 1978; Speirs and Gurney 2001; see also Fig 6). **(f)** Recolonization of downstream patches will occur via hydrochory, recolonization of empty upstream patches via zoochory (white dots signify suitable but empty patches, gray dots occupied patches).

clearly different features: *e.g.* (i) free flowing versus fragmented rivers (*e.g.* Jansson *et al.* 2005; and references therein) (ii) straight canalized rivers versus meandering rivers, (iii) upland river stretches versus lowland river stretches, and (iv) small streams versus large river systems. Finally, up till now most studies on plant dispersal on rivers have used an empirical approach. We suggest that future research on plant dispersal in rivers would benefit most when applying, and comparing the outcome of, different approaches (*i.e.* empirical, mechanistic and molecular approaches).

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Summary

The plant's ability to disperse its generative and vegetative propagules to other locations is essential for offspring survival (avoidance of disproportionate seedling mortality due to sibling competition near the parent plant), population viability (avoidance of inbreeding depression by exchanging genetic information between populations), metapopulation persistence (by continuous recolonisations of depopulated habitat patches), and range expansion of the species (particularly important in biological invasions).

While dispersal is a very important biological process, it is also very difficult to quantify. Three different approaches have been used to quantify plant dispersal, i.e. the empirical, mechanistic and molecular approaches. Empirical approaches assess the amount and distance of seed dispersal directly in the field, by means of trapping seeds, seed mimics or vegetative propagules at various distances from the source plants. Mechanistic approaches assess the dispersal characteristics of seeds under controlled (experimental) conditions and relate this information to the putative dispersal agents in order to construct predictive (mathematical) models of seed dispersal. Molecular approaches assess the distribution of genetic variation within and among populations, in order to make inferences about the rate of gene flow that has occurred between them. In this thesis we used a mechanistic and a molecular approach to study plant dispersal in river systems.

In **chapters 2, 3 and 4** we applied a mechanistic (experimental) approach, using seed-feeding-experiments, to examine the factors that affect the dispersal of seeds by fish (ichthyochory) and waterfowl (ornithochory). In **chapter 2** we show that intraspecific variation in seed size within the unbranched bur-reed (*Sparganium emersum*) has little or no affect on the probability of dispersal or on the potential dispersal distance of differently sized seeds when dispersed by the common carp (*Cyprinus carpio*). In **chapter 3** we show that interspecific variation in seed morphology between *S. emersum* and arrowhead (*Sagittaria sagittifolia*) affects the probability of dispersal (being higher for *S. emersum*), but does not affect the potential dispersal distance of both plant species, when dispersed by the common carp. In **chapter 4**, however, we show that, when dispersed by teal (*Anas crecca*) and mallard (*Anas platyrhynchos*), interspecific variation in seed morphology between *S. emersum* and *S. sagittifolia* affects both the probability of dispersal as well as the potential dispersal distance (also higher for *S. emersum*). This difference between carp (Class Osteichthyes) on the one hand, and teal and mallard (Class Aves) on the other, is most likely related to the large differences in their digestive systems. In **chapter 4** we furthermore show that there are no, or very little, differences in the probability and potential distance of seed dispersal between teal and mallard, despite the large interspecific differences in body weight between these two waterfowl species. This lack of difference is most likely due to the fact that these two closely related *Anas* spp. have very similar digestive physiologies. Finally, in **chapter 4** we introduce the concept of the ´Drift-paradox for plants in river systems´, and argue that animal-mediated dispersal in an upstream direction may be important for the persistence of plant populations in rivers.

In **chapters 5, 6 and 7** we applied a molecular (population genetic) approach, using

neutral genetic markers (e.g. microsatellites and AFLPs), to examine the reproductive strategy, gene flow and genetic structure of *S. emersum* in three different river systems. In **chapter 5** we describe the development and characterization of 6 novel microsatellite markers for *S. emersum*, as well as their potential for cross-species amplification in the related species, branched bur-reed (*S. erectum*). In **chapter 6** we applied these microsatellites to genotype *S. emersum* plants collected from populations in the Swalm and Rur rivers. We show a striking difference in genotypic diversity within populations, between both rivers. We furthermore show that this difference in genotypic diversity is related to differences in the locally reigning hydrodynamic conditions in both river systems. These differences in hydrodynamic conditions affect the morphology of *S. emersum* plants inhabiting these river systems and, in turn, affect their mode of reproduction (exclusively asexual in the Swalm, but both sexual and asexual in the Rur). In **chapter 7** we propose a number of testable predictions about the genetic structure of populations and the rate of gene flow between them, to help identify regional plant population structures in the field (i.e. spatially extended populations, metapopulations and regional ensembles). In **chapters 6 and 7** we used these testable hypotheses to examine the regional population structure of *S. emersum* in Swalm and Rur rivers (**chapter 6**) and the Niers river (**chapter 7**). We show that there are large differences in plant population structure between these three river systems. In the Rur River *S. emersum* is more likely to act as a spatially extended population, whereas in the Niers River *S. emersum* appears to exist as a metapopulation. The monoclonal *S. emersum* 'populations' of the Swalm River do not fit into any of the regional population structure models described by Freckleton & Watkinson, since these are single individual plants rather than true populations.

In **chapter 8** the results obtained in this thesis are put into a broader perspective. Our findings contribute to a deeper insight into the various mechanisms of plant dispersal in rivers, and present novel views on their importance for plant population persistence in river systems. Moreover, we caution against generalizations on the dispersal among, and the regional population structures of, plant populations inhabiting different river systems.

Samenvatting

Het vermogen van planten om hun generatieve en vegetatieve diasporen naar andere locaties te verspreiden (dispersie) is essentieel voor de overleving van hun nakomelingen (middels het vermijden van sterfte onder zaailingen nabij de moederplant ten gevolge van onderlinge competitie), hun populaties (middels het vermijden van inteeltdepressie door middel van het uitwisselen van genetische informatie tussen populaties) en hun metapopulaties (middels continue rekolonisaties van lege habitat plekken), en speelt een belangrijke rol bij de uitbreiding van het leefgebied van een soort tijdens een biologische invasie.

Hoewel dispersie een zeer belangrijk biologisch proces is, is het tegelijkertijd ook een buitengewoon moeilijk te bestuderen proces. Er zijn drie benaderingen die vaak worden toegepast om het proces van dispersie te bestuderen, namelijk de empirische, mechanistische en moleculaire benaderingen. In de empirische benadering onderzoekt men de hoeveelheid en afstand van zaadverspreiding direct in het veld, met behulp van zaadvallen die op verschillende afstanden van de moederplanten worden geplaatst en waarin zowel generatieve als vegetatieve diasporen kunnen worden gevangen. In de mechanistische benadering bestudeert men specifieke zaadkarakteristieken die de verspreiding van deze zaden beïnvloeden, relateert deze gegevens aan informatie over de vectoren die deze zaden verspreiden (wind, water, dieren), en construeert vervolgens op basis van deze gegevens voorspellende (mathematische) modellen voor de verspreiding van deze zaden. In de moleculaire benadering onderzoekt men de verspreiding van genetische variatie, zowel tussen als binnen populaties, en trekt hieruit vervolgens conclusies over de richting, frequentie en afstand van gene flow (het genetische equivalent van dispersie) tussen populaties. In dit proefschrift hebben we de mechanistische en moleculaire benadering toegepast om de verspreiding van planten in riviersystemen te bestuderen.

In **hoofdstuk 2, 3 en 4** hebben we de mechanistische (experimentele) benadering gebruikt om zaadverspreiding door dieren (zoöchorie) te bestuderen. Hiertoe hebben we voeder-experimenten uitgevoerd om de factoren te onderzoeken die een rol spelen bij de zaadverspreiding door vissen (ichthyochorie) en watervogels (ornithochorie). In **hoofdstuk 2** laten we zien dat *intra*specifieke variatie in zaad grootte in de kleine egelskop (*Sparganium emersum*) weinig tot geen effect heeft op de waarschijnlijkheid van verspreiding noch op de potentiële verspreidingsafstand van zaden van verschillende grootte, wanneer ze verspreid worden door de karper (*Cyprinus carpio*). In **hoofdstuk 3** zien we dat *inter*specifieke variatie in zaadmorfologie tussen *S. emersum* and pijlkruid (*Sagittaria sagittifolia*) de waarschijnlijkheid van verspreiding beïnvloedt (de waarschijnlijkheid van verspreiding is groter voor *S. emersum*), maar geen effect heeft op de potentiële verspreidingsafstand van beide plantensoorten, wanneer ze verspreid worden door de karper. In **hoofdstuk 4** zien we vervolgens dat, wanneer beide plantensoorten verspreid worden door de wintertaling (*Anas crecca*) en de wilde eend (*Anas platyrhynchos*), de *inter*specifieke variatie in zaadmorfologie tussen *S. emersum* en *S. sagittifolia* zowel een effect heeft op de waarschijnlijkheid van verspreiding (groter voor *S. emersum*) als op de potentiële verspreidingsafstand (ook groter voor *S. emersum*). Dit verschil

tussen de karper (Klasse Osteichthyes) enerzijds, en de wintertaling en wilde eend (Klasse Aves) anderzijds, is gerelateerd aan de grote verschillen in de morfologische, chemische en mechanische eigenschappen van hun verteringsstelsels. In **hoofdstuk 4** laten we bovendien zien dat er weinig tot geen verschillen zijn in de waarschijnlijkheid van verspreiding en de potentiële verspreidingsafstand tussen zaden die verspreid worden door enerzijds wintertaling en anderzijds wilde eend, ondanks the grote *inter*specifieke verschillen in lichaamsgewicht tussen deze twee eendensoorten. Dit gebrek aan verschil is zeer waarschijnlijk het gevolg van het feit dat deze twee nauw verwante *Anas* spp. beide een zeer vergelijkbare verteringsfysiologie hebben. Tenslotte introduceren we in **hoofdstuk 4** het concept van de ´Drift-paradox voor planten in rivieren´, en beargumenteren dat zaadverspreiding door dieren in een stroomopwaartse richting belangrijk zou kunnen zijn voor de overleving en instandhouding van planten populaties in rivier-systemen.

In **hoofdstuk 5, 6 en 7** hebben we een moleculaire (populatie genetische) benadering toegepast om de wijze van voortplanting, de gene flow en de genetische structuur van *S. emersum* te onderzoeken in drie verschillende rivier-systemen. In **hoofdstuk 5** beschrijven we de ontwikkeling van zes nieuwe microsatelliet markers voor *S. emersum*, alsmede hun potentiële amplificatie in de nauw verwante plantensoort, de grote egelskop (*S. erectum*). In **hoofdstuk 6** zijn deze microsatellieten vervolgens toegepast om *S. emersum* populaties uit de Swalm en de Rur te analyseren. We tonen een opmerkelijk verschil in genotypische diversiteit binnen populaties, tussen beide rivieren. Daarna beschrijven we grote verschillen in de locale hydrodynamische condities (met name watersnelheid), alsmede grote verschillen in de morfologie van *S. emersum* planten (groeivorm, biomassa en dichtheid van scheuten) tussen beide rivieren. Vervolgens beargumenteren we dat de verschillen in hydrodynamische condities tussen beide rivieren een grote invloed uitoefenen op de morfologie van *S. emersum* planten, dat deze morfologie vervolgens de wijze van voortplanting bepaalt (sexueel vs klonaal), en dat de wijze van voortplanting uiteindelijk belangrijke gevolgen heeft voor de genotypische diversiteit binnen populaties. In **hoofdstuk 7** presenteren we een aantal aannames over de gene flow en de genetische structuur van populaties, die mogelijk gebruikt kunnen worden om de regionale structuur van planten populaties in het veld vast te stellen (e.g. ´spatially extended populations´, ´metapopulations´ en ´regional ensembles´, *sensu* Freckleton & Watkinson 2002). In **hoofdstuk 6 en 7** gebruiken we deze aannames om de regionale populatie structuur van *S. emersum* in de Swalm en de Rur (**hoofdstuk 6**) en de Niers (hoofdstuk 7) te onderzoeken. Uit beide hoofdstukken blijkt dat er grote verschillen bestaan in de regionale populatie structuur van *S. emersum* tussen deze drie rivieren. In de Rur vormen de subpopulaties van *S. emersum* zeer waarschijnlijk één grote ´spatially extended population´, terwijl ze in de Niers waarschijnlijk een metapopulatie structuur vormen. De monoklonale ´populaties´ in de Swalm passen eigenlijk in geen enkel van de regionale populatie structuur modellen die door Freckleton & Watkinson zijn beschreven, aangezien we hier niet te maken hebben met echte populaties, maar met groepen planten die bestaan uit één enkel individu (één genet bestaande uit verschillende ramets).

In **hoofdstuk 8** zijn de verkregen resultaten in een breder perspectief geplaatst. Onze bevindingen dragen bij aan een beter inzicht in de verscheidene mechanismen van plantverspreiding in rivier-systemen, en leveren nieuwe inzichten over hun respectievelijke bijdrage aan de overleving en instandhouding van planten populaties in rivieren. Daarnaast blijkt uit dit proefschrift dat men

voorzichtig moet zijn bij het maken van generalisaties over de regionale structuur van, en de dispersie (connectiviteit) tussen, plant populaties in rivieren, aangezien deze sterk kunnen verschillen tussen verschillende rivier-systemen.

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List of Publications

Peer reviewed publications

Pollux BJA, Van der Velde G & Bij de Vaate A (2007) A perspective on a global range expansion by the zebra mussel (*Dreissena polymorpha*): A review on possibilities and limitations. In: *Zebra mussels in Europe* (eds Van der Velde G, Rajagopal S & Bij de Vaate A). Backhuys Publishers BV, Leiden, in press.

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Curriculum vitae

Bartholomeus Adrianus Johanna Pollux was born on January 19, 1973 in Sittard, the Netherlands. In 1990 he received his HAVO degree, and in 1993 his Athenaeum degree, at the St. Thomascollege in Venlo. That same year he started his studies in biology at the University of Nijmegen. During his B.Sc. studies Bart was an active member of the student biology association (BeeVee), and travelled, lived and worked in several places around the world. During his first M.Sc. thesis he studied the nursery function of mangroves, seagrass beds and shallow reef flats for coral reef fishes on the Caribbean island of Curaçao (Netherlands Antilles); and during his second M.Sc. thesis he investigated the origin of invasion by the zebra mussel (*Dreissena polymorpha*) to Ireland. In April 2001 he graduated with honors (*Cum laude*) in biology at the University of Nijmegen. His PhD project on plant dispersal in rivers, presented in this thesis, was started that same month at the Department of Aquatic Ecology & Environmental Biology (Radboud University Nijmegen) in collaboration with the Department of Plant-Animal Interaction (Centre of Limnology, Netherlands Institute of Ecology, NIOO-KNAW). The results obtained during this project have been published in international peer-reviewed journals and presented at various international scientific conferences.